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(54) Title: METHODS FOR TRANSDUCING FUSION MOLECULES

(57) **Abstract**

The present invention provides highly efficient methods of transducing a desired cell or group of cells including tissue or an organ with a fusion molecule, the method comprising, misfolding the fusion molecule; and contacting the misfolded fusion molecule to the cell under conditions sufficient to transduce the fusion molecule into the cell.

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METHODS FOR TRANSDUCING FUSION MOLECULES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to highly efficient methods for transducing fusion molecules into cells or groups of cells. The present methods have a variety of uses including transducing therapeutic fusion molecules into cells with high efficiency.

2. Background

There has been significant interest in developing methods that can provide cells, tissues and organs with heterologous protein. A majority of methods for achieving this goal have focussed on introducing nucleic acid into cells and then expressing that nucleic acid to generate the protein. See generally, Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.); and Ausubel et al., *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York.

However, many cells are essentially impermeable to nucleic acids. That recognition has led to attempts to improve methods of transferring nucleic acids and other macromolecules into the cells.

For example, one standard method involves making cell holes and then transferring the nucleic acid through the holes e.g., by diffusion or along an imposed gradient. The holes can be made by a variety of means such as physical penetration. Particular examples of such transfer methods have been disclosed. See e.g., Cockett, et al., *Bio/Technology* 8:662 (1990); Ausubel et al., *supra*; and Sambrook et al., *supra*.

Other nucleic acid transfer methods employ a bacteriophage or a virus to introduce the nucleic acid into cells by infection methods sometimes referred to as "transformation" or "transfection" methods. Once inside the target cell, the nucleic acid is

typically expressed as part of the bacteriophage or virus lytic cycle. See e.g., Ausubel et al., *infra*; and Sambrook et al., *infra*.

However, the prior methods of introducing macromolecules and particularly nucleic acids into cells has been associated with significant drawbacks.

For example, most transformation methods are optimized to transfer nucleic acids within limited size ranges (generally about 20 to 50 kB or less). Transformation of larger nucleic acids is not always successful.

In addition, many methods of transferring nucleic acids into cells have been reported to be associated with undesired properties, e.g., nucleic acid over-expression, non-uniformity in levels of expressed protein, and low percentage of cells targeted by the methods. See e.g., Chen et al. *Mol. Cell Biol.* 7, 2745-2752 (1987). Friedman, T. *Nat. Gene* 2:93 (1992). Bar-Sagi, D. *Meth. Enzy.* 255,436-442 (1995).

Further, pharmacologically relevant peptidyl mimetics and/or proteins have a size constraint problem that limits bioavailability when in excess of about 1 kDa.

There have been attempts to develop alternative methods of introducing macromolecules into target cells, tissues and organs. One approach involves genetically fusing amino acid sequences to a specified transfer molecule and then introducing the resulting construct fusion into cells.

For example, one specific approach has attempted to transport proteins into cells by genetically fusing the proteins to specified transducing proteins. The transducing proteins have been reported to carry the resulting fusion molecules into target cells by a process referred to as protein "transduction". See e.g., Frankel, A.D. and C.O. Pabo (1988) *Cell* 55:1189; Fawell, S. et al. (1994) *PNAS (USA)* 91: 664; Chen, L.L. et al. *Anal. Biochem.*, 227:168 (1995); U.S. Pat. No. 5,652,122.; and PCT WO/04686.

However, the prior transduction methods have been associated with significant problems that have negatively impacted widespread use of the methods.

For example, many prior transduction methods are not capable of transducing sufficient amounts of fusion proteins into target cells. Intracellular amounts of the fusion protein and their biological activities can therefore be limited. Attempts to overcome these and related problems have included transducing large amounts of fusion proteins into target cells. However, that strategy can sometimes be lethal to transduced cells. In addition, rates of transduction exhibited by prior transduction methods often have been less than optimal.

Further, the prior transduction methods are usually optimized to maintain fusion proteins in a native confirmation. Such methods can be complicated by attempts to maintain that native confirmation during use or storage of the fusion protein.

It would thus be desirable to have more efficient methods of transducing desired fusion molecules and particularly, fusion proteins into cells.

SUMMARY OF THE INVENTION

The present invention features highly efficient methods for transducing a fusion molecule into cells or groups of cells. The methods generally include misfolding the fusion molecule and contacting the misfolded fusion molecule to the cells under conditions sufficient to transduce the misfolded fusion protein. Further provided are misfolded fusion molecules and methods for making same. The present invention has a variety of uses including providing highly efficient transduction of therapeutic fusion molecules *in vitro* or *in vivo*.

It has been discovered that misfolded fusion molecules can be transduced into cells much more effectively than native fusion molecules. More particularly, it has been

found that a misfolded fusion molecule can transduce by as much as about one to three orders of magnitude more efficiently than the same fusion molecule in its native state (conformation). A fusion molecule is "misfolded" in accord with this invention if it has been at least partially denatured and refolded and has decreased biochemical and/or biological activity as described herein.

The present invention can be used to increase transduction of a wide spectrum of fusion molecules. For example, the present methods can be used to lower an effective amount of a therapeutic fusion molecule by as much as about 2, 3, 5, 10, 50 or 100-fold or more compared to the effective amount required by the prior transduction methods, while achieving about the same degree of protein transduction, (e.g., number of molecules transduced per cell). Importantly, the lower effective amount does not impact the therapeutic activity of the fusion molecule in the transduced cells. That is, the present invention affords the same or nearly the same therapeutic activity in the transduced cells even when substantially lower amounts of the fusion molecule are used. As will be discussed in more detail below, the invention achieves these objectives by misfolding the fusion molecule, thereby significantly boosting the transduction efficiency.

The present invention particularly enhances the transduction efficiency of large therapeutic fusion molecules such as those comprising one or more full-length proteins.

The present invention features transduction methods that are not limited to use of any particular fusion molecule, target cell or group of such cells. Thus, nearly any fusion molecule that has been misfolded in accord with this invention can be used to transduce a wide variety of cells *in vitro* or *in vivo*.

A fusion molecule can be misfolded in accord with this invention by one or a combination of strategies. For example, the fusion molecule can be misfolded by exposing the molecule to a first condition that is sufficient to partially or completely

unfold (i.e., denature) the fusion molecule. The denatured molecule is then further exposed to a second condition that is conducive to misfolding the fusion molecule.

The misfolded fusion molecule can be provided by one or more methods. In one approach, the first condition for denaturing the fusion molecule comprises contacting the fusion molecule with one or more protein denaturing agents. In most cases, the fusion molecule is present in its native or near-native conformation prior to exposure to the first condition. Also in most cases, the native or near-native fusion molecule is energetically stable and has, at least in theory, a low Gibbs free energy (ΔG) in relation to the misfolded fusion molecule.

A suitable denaturant is selected to provide partial or complete denaturation of the fusion molecule. Compositions and conditions that denature proteins are known to those in the art (see below). That is, the denaturing agent is typically selected to disrupt intramolecular bonds such as hydrogen and ionic bonds which themselves contribute to the secondary, tertiary or quarternary structure that the protein conforms to in solutions or buffers that are composed of physiological concentrations of salts, and other components, "physiological" being defined as a solvent composition that mimics in certain respects the natural solvent for proteins and polypeptides, namely the cell cytoplasm, blood, plasma and interstitial fluids. The denaturing agent usually will not have substantial capacity to bind the fusion molecule, although such a denaturing agent can be used if it can be substantially separated from the denatured fusion molecule during purification. Illustrative denaturing agents include at least one of an organic compound, a salt, a detergent, temperature or sound.

Without wishing to be bound to any particular theory, it appears that by increasing the Gibbs free energy (ΔG) of the denatured fusion molecule, it is possible to misfold the fusion molecule, thereby making a fusion molecule exhibiting significantly enhanced transduction capacity. It will therefore be desirable to expose the denatured fusion molecule to a second condition that is preferably selected to increase the energetic

instability of the denatured molecule. That increase in energetic instability is believed to be achieved by contacting the denatured molecule with a solution that preferably increases the energetic instability of the denatured fusion molecule. Illustrative are solutions that increase the Gibbs free energy (ΔG) of the denatured fusion molecule by at least about 2 to 100 fold up to about 1000 fold or more compared to the Gibbs free energy of the native or near-native fusion molecule. Preferred are solutions that significantly reduce or eliminate presence of the denaturing agents contacting the denatured molecule. More preferred aqueous solutions and particularly aqueous buffers such as those disclosed herein.

Sometimes exposure of the denatured fusion molecule to the second condition is referred to herein as "shock" misfolding to denote that exposure.

As noted, the denatured fusion molecule is subjected to the second condition to form the misfolded fusion molecule. In one embodiment, that second condition is achieved by contacting the denatured molecule with an aqueous buffer (or more than one aqueous buffer if desired) sufficient to form the misfolded fusion molecule. The contact can be suitably accomplished by a variety manipulations. For example, the denatured molecule can be applied to at least one chromatographic implementation. Alternatively, the denatured molecule can be precipitated and then suspended into the aqueous buffer, or the denatured molecule can be filtered into the aqueous buffer. In cases where it is desired to use a chromatographic implementation to effectuate the contact, one or more suitable eluting solutions are used. Generally, those eluting solutions include or consist of the aqueous buffer. Preferred eluting solutions are usually capable of releasing the fusion molecule (denatured or misfolded) from the chromatographic implementation used.

Manipulations that effectuate rapid desalting of the denatured fusion molecule, from the denaturing solution into an aqueous solution, e.g., by use of an appropriate chromatographic implementation, are particularly preferred for many applications.

As noted, the second condition is preferably selected to enhance the energetic instability of the denatured fusion molecule sufficient to form the misfolded fusion molecule into an aqueous buffer. Thus, manipulations which are not optimized to substantially increase that instability are not usually preferred.

In another embodiment of the invention, the contact between the denatured fusion molecule and the aqueous buffer (or more than one aqueous buffer) can be accomplished by diluting the denatured fusion molecule into the aqueous buffer. Generally, the dilution will be conducted in a ratio of between about 1:10 (v/v) to 1: 100,000 (v/v) or more depending on intended use. The diluted (and misfolded) fusion molecule can be concentrated if desired by several conventional means such as membrane filtration or precipitation.

The present invention also features substantially pure preparations of misfolded fusion molecules that are suitable for a variety of uses including pharmaceutical or research use. Preferably the misfolded fusion molecule is provided as a sterile composition which can include other additives as desired. For example, the misfolded fusion molecule may include one or more of the denaturing agents to enhance or maintain the misfolding, e.g., during storage. In addition, the misfolded fusion molecule may include one or more stabilizing agents such as those known to be useful in the stabilization of proteins such as certain albumins, glycols, and sulfoxides.

The present invention provides several important advantages. For example, it has been discovered that by using the misfolded fusion molecules, transduction efficiency can be increased by up to about 2, 5, 10, 20, 50, 100, or 200 fold or more when compared to transduction with a suitable control molecule. A preferred control molecule is the native fusion molecule, i.e. the fusion molecule that has not been treated to produce a misfolded, higher energy confirmation. The present transduction methods are thus much more efficient than most prior transduction methods using native or near-native fusion

molecules. As will be explained in more detail below, the enhanced transduction efficiency is evidenced by several parameters such as transduction rate and biological activity of transduced fusion protein.

As noted, the present methods provide highly efficient transduction of a variety of fusion molecules including fusion proteins and particularly large fusion proteins such as those potentially exhibiting therapeutic activity. Lower effective amounts of the misfolded fusion molecules can be used to transduce cells or groups of cells, thereby significantly reducing or eliminating toxicity to the transduced cells.

The methods of the present invention provide further advantages. For example, the transduction methods described herein provide for transduction of fusion proteins, the size of which varies over a large range, including large proteins e.g. proteins having a molecular weight of about 5, 10, 20, 30, 40, 50, 100, 200, 300, 400 or 500 kDa or more. Transduction of smaller fusion proteins is also readily accomplished by the present methods. In contrast, most prior transduction methods are optimized to transduce only the smaller fusion proteins. Widespread use of the prior methods has therefore been constrained by the size of the protein to be transduced. In particular, it has been difficult to transduce many therapeutic proteins and particularly large therapeutic proteins by the prior transduction methods.

Still further, the present invention avoids the need to miniaturize important protein domains (e.g., active or contact sites). Prior methods generally have been limited to transducing miniaturized protein domains (fragments) and/or peptidyl mimetics into desired target cells. However, it is recognized that protein domains can be large, sometimes exceeding about 15 up to 500 kDa or more in size. The present invention thus overcomes such limitations of prior practice and provides methods for the efficient transduction of very large (and intact) proteins into cells. Additionally, cost and time associated with miniaturizing the protein domains is minimized or even avoided by the present invention.

The methods of the invention also positively impact purification and yields of the fusion molecules. For example, preferred methods include use of one or more denaturing agents to purify fusion proteins from cell components which naturally accompany it. Use of those denaturing agents helps to free the fusion protein from the cell components, such as denatured and insoluble proteins that generally comprise the bulk of recombinant proteins and often present in bacterial inclusion bodies, thereby increasing both purity and yield substantially.

An illustrative preparative strategy according to the present invention involves expressing a desired fusion protein in host cells and isolating that fusion protein from fractions obtained from those cells. Isolation of the fusion proteins from the fractions and particularly insoluble fractions, has several advantages including protecting the misfolded fusion protein from degradation by host cell proteases. Significantly, preferred use of denaturing agents to prepare misfolded fusion proteins helps avoid time-consuming and costly protein renaturation techniques.

The methods of the invention also are generally flexible and can be tailored to provide quantities of a misfolded fusion molecule as needed. Specific methods include preparing fusion proteins in amounts ranging over a large scale (i.e., from microgram to gram to kilogram quantities) from a variety of implementations including roller bottles, spinner flasks, tissue culture plates, bioreactor, or a fermentor. The large-scale fusion protein preparation can then be misfolded according to any of the methods described herein.

As noted above, the methods of the invention can be used to enhance transduction of a desired fusion molecule into target cells or groups of such cells. Accordingly, preparation and use of large amounts of the fusion molecule can be avoided if desired. This feature is particularly useful in situations where a given fusion molecule is difficult

to prepare, or where large amounts of the fusion molecule are damaging to target cells *in vitro* or *in vivo*.

The present methods are also useful for producing misfolded fusion molecules in large quantities, e.g., to provide the fusion molecule as one component of a kit suitable for medical, research, home or commercial use. Further, it is useful to have methods of producing certain misfolded fusion molecules on a large-scale, e.g., to simplify structural analysis of the fusion molecule, to assist in the kinetic analysis of transduction, as well as for further purification and/or testing if desired.

The misfolded fusion molecules of this invention have a variety of additional uses including delivering a tag (marker) such as fluorescent, phosphorescent or immunological tag to a cell or group of cells. Alternatively, or in addition, the misfolded fusion molecules can be used to deliver a specified activity to the cells such as therapeutic activity. The misfolded fusion molecules can be used *in vitro* or *in vivo* as needed.

Examples of therapeutic activity include delivery of therapeutic molecules including one or more radionuclides or cytotoxic drugs.

As noted, the present invention is not limited to the preparation or use of a particular type of fusion molecule. Illustrative fusion molecules generally include a transduction domain covalently linked to one or more desired molecules. Preferred fusion molecules generally include a transduction domain and an amino acid sequence covalently linked as a genetic in-frame fusion protein. In most cases the fusion protein will be made by standard genetic manipulations although in some cases it will be useful to make certain fusion molecules by chemical cross-linking as discussed below. The molecular weight of the fusion molecule can be up to about 50 to 500 kD or more as estimated by SDS-PAGE gel electrophoresis or sedimentation centrifugation.

Exemplary transduction domains include TAT, Antp, or specified transducing portions thereof such as the TAT basic region. Also contemplated are transduction domains comprising synthetic peptides. See e.g., Frankel, A.D. and Pabo, C.O., *supra*; Falwell, S., et al. *supra*; Chen, et al. *supra*; U.S. Pat. Nos. 5,652,122 and PCT WO/04686.

As noted above, the misfolded fusion molecules of this invention are preferably used to transduce desired cells or groups of such cells including tissue and organs. The methods can be used *in vitro*, e.g., with a culture of primary or immortalized cells, or *in vivo* if desired.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a drawing showing the pTAT expression vector.

Figure 1B is also a drawing of the pTAT expression vector illustrating a protein "X" fused to 6XHIS (HIS tag) and TAT.

Figure 2 shows nucleotide and amino acid sequences of pTAT linker and pTAT HA linker. A minimal TAT domain is in bold. Underlined sequence designates the minimal TAT domain flanked by glycine residues.

Figure 3 is a drawing outlining a purification and "shock" misfolding protocol. The triangle indicates application of an imidazole concentration ramp. "Desalt" indicates desalting of the shock misfolded fusion protein.

Figure 4 is a representation of an SDS-PAGE gel stained with Coomassie Blue. The gel shows various fractions obtained from purification of TAT-p16^{wt} using the protocol outlined in Fig. 3. Ss = start, FT = flow through.

Figure 5 is a representation of an SDS-PAGE gel stained with Coomassie Blue. The gel compares TAT-p 16^{wt} before (lane 1) and after (lane 2) freeze-thaw of sample stored in 10% glycerol and frozen at -80 C.

Figures 6A-F are histograms obtained by analyzing FITC-labeled TAT fusion proteins using fluorescence activated cell sorting (FACS). FITC-labeled TAT-$p16^{wt}$ (6A) and TAT-HSV TK (6B) fusion proteins were added to Jurkat T cell cultures and analyzed by FACS at 40 and 160 min post addition. FITC-labeled TAT-HSV TK fusion protein was added to human whole blood (6C) or large, nucleated blood cells (6D) were analyzed by FACS at 1 hr post addition. Equilibrium FACS analysis of Jurkat cells 1 hr post transduction with 0.25, 1.25, 10 nM TAT-p 16^{wt}-FITC protein (6E) or 4, 16, 40 nM TAT-HSV-TK-HTC fusion protein (6F).

Figures 7A, B are photomicrographs showing confocal microscopy of Jurkat T cells transduced with control (7A) or TAT-p 16^{wt}-FITC protein (7B).

Figure 8 A and 8B are representations of an SDS-PAGE gel showing formation of *in vivo* complexes of TAT-$p16^{wt}$ with Cdk6 and TAT-I 35 El A with pRb. p16(-l-) Jurkat T cells were transduced with 10 and 200 nM of TAT-$pl6^{wt/mut}$ fusion proteins during ^{35}S-methionine labeling of cellular proteins for 4 hr (8A). Keratinocytes were labeled with ^{35}S-methionine in the presence of 100 nM TAT-13SE1A^{mut} or 100 nM TAT- 13S ElA^{wt} fusion proteins, treated as above and immunoprecipitated with anti-E7 or anti-ElA antibodies followed by anti-pRb antibodies and SDS-PAGE resolution (8B). The positions of Cdk6 and pRb are as indicated. The symbol "α" followed by a protein designation indicates treatment with an antibody specific for that protein. "IP" indicates immunopurification using $\alpha p16^{wt}$ or αcdk6 antibodies and "mut" denotes $p16^{mut}$ protein.

Figures 9A-E are graphs illustrating G1-phase specific arrest of p16(-) Jurkat T cells by transduction of TAT-$p16^{wt}$ protein.

Figure 10A is a schematic drawing showing misfolded fusion proteins and native fusion protein adjacent to a Gibbs free energy (ΔG) diagram. The misfolded fusion proteins are energetically unstable relative to the native fusion protein.

Figure 10B is a model illustrating enhanced transduction of misfolded fusion protein relative to its native protein conformation. In this model, the transduced misfolded fusion protein is renatured in the transduced cell by a chaperon Hsp 90.

Figures 11A-11F are representations of photomicrographs showing that a misfolded TAT-p27^{wt} fusion protein is capable of facilitating cell movement. TGF-β = transforming growth factor-beta; HGF = hepatocyte growth factor.

Figure 12 is a graph showing percentage of cell migration following transduction with TAT-p27^{wt} in soluble and native folded form (SL) versus denatured and misfolded (DN) form.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention features highly efficient methods for transducing a fusion molecule into a desired cell or group of cells. The methods generally involve denaturing the fusion molecule and then subjecting the denatured fusion molecule to conditions specified herein. The misfolded fusion molecule is then contacted to cells or groups of cells of interest to transduce the fusion molecule into those cells. As noted, the present methods are highly efficient and are capable of substantially enhancing transduction efficiency of a variety of fusion molecules. Further provided are misfolded fusion molecules and methods of making those misfolded fusion molecules.

Transduction efficiency can be measured by one or a combination of different strategies. For example, the transduction efficiency as a given fusion protein can be measured by monitoring the rate at which that fusion protein can be measured by

monitoring the rate at which that fusion protein enters cells or group of cells. A more preferred method of measuring transduction efficiency is by determining the biological activity of the fusion protein once inside the cell. Biological activity can be measured by a variety of ways provided herein.

Preferred misfolded fusion molecules of this invention can transduce at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, preferably at least about 90%, and more preferably at least about 95% or even about 100% of the total number of targeted cells as measured by assays specified below.

The transduction efficiency of a misfolded fusion molecule of interest can be measured and quantified if desired by a variety of conventional approaches. In general, the approaches involve measuring parameters of transduction with reference to a suitable control molecule. In most cases, the control molecule will be transduced under the same or nearly the same conditions as the misfolded fusion molecule. Generally, the control molecule will be the fusion molecule in its native conformation, i.e., not having been exposed during its isolation to denaturation and "shock" misfolding conditions described herein. In some cases it will be useful to compare transduction efficiency of the misfolded fusion molecule to two control molecules particularly the native and near-native forms of fusion molecule. The transduction efficiency can be expressed in a variety of ways including the percent increase in the efficiency relative to transduction with a desired control molecule.

By the term "near-native" is meant that the fusion molecule has been denatured by exposure to a denaturing agent followed by substantial renaturation. The denatured fusion molecule is typically renatured by dialysis into a physiologically acceptable buffer such as phosphate buffered saline (PBS). Many, but not all, of the intramolecular bonds present in the renatured fusion molecule (e.g., hydrogen and ionic bonds) will be the same or similar to the native molecule. Such fusion molecules are also sometimes referred to herein as "denatured dialyzed" fusion molecules.

By the term "native" is meant that protein shape and/or structure resulting from contact with an essentially physiological environment, e.g., the cell cytoplasm and/or a physiologically acceptable buffer such as buffered saline. In contrast, by the term "denatured" is meant that protein shape and/or structure resulting from contact with an essentially chaotropic environment, e.g., exposure to one or more of the denaturing agents described herein. Most protein molecules described herein will retain substantial activity in a native shape but will lose most or all of that activity when denatured, e.g., from about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or greater activity, when compared to the corresponding native molecule. Denaturation can include e.g. unfolding of the native molecule sufficient to transform the native protein into a random loop (coil) configuration. Denaturation can be monitored by a number of recognized methods including measuring protein activity, conducting viscosity measurements, and/or analyzing optical rotation spectra.

By the term "increased efficiency" or similar term as it relates to transduction of a desired fusion protein is meant at least one of: 1) increasing the percentage of molecules that are in contact with the target cells that are delivered into the cell, 2) increasing the rate of protein transfer into target cells, 3) increasing the percentage of cells exposed to the protein that accumulate the protein, 4) increasing refolding in situ or inside cells, and 5) increasing biological activity of the protein.

As noted, transduction efficiency can be measured by monitoring the rate of which a desired misfolded fusion molecule enters a cell or, more preferably, measuring biological activity of the protein.

The rate determination is typically conducted by standard kinetic analysis using one or more suitable control molecules. A preferred misfolded fusion proteins is capable of transducing target cells faster than the control molecule, generally about 2, 3, 4, 5, 10, up to about 100 times faster than a native control molecule. Particularly preferred

misfolded fusion proteins exhibit maximum intracellular concentrations in transduced cells in less then about 1 to 2 hours, more preferably less than an hour, and still more preferably between about 5, 10, 15, 20, 30, 35, up to about 40 to 50 minutes. The analysis is usually conducted by a suitable microscopic or cell sorting technique as described below. In most cases, determining rates of transduction will be useful for fusion molecules having a molecular weight of at least about 50, 60, 70, 80, 90, 100 or more kDa.

Additional assays can be conducted that measure levels of the fusion molecule inside transduced cells compared to a suitable control molecule. Specified methods for determining intracellular levels of the fusion molecules in transduced cells generally involve conventional techniques of protein detection in living cells, including but not limited to Western immunoblotting of cell extracts using antibodies to the protein of interest, microscopy, e.g., fluorescence microscopy, or cytometric determinations using fluorescence activated cell sorting (FACS). Typically, the control molecule is detectably-labeled to assist in the measurements, e.g., by attaching a detectable tag. Examples of suitably detectable tags include certain amino acid sequences and include those specified fluorescent proteins described below. Particularly preferred misfolded fusion molecules of this invention are capable of increasing the intracellular levels of transduced fusion molecule by about 2, 5, 10, 20, 30, 50, 70, 100, 200 or more fold when compared to a suitable native control molecule.

A preferred assay for measuring the transduction efficiency is monitoring the biological activity of the fusion molecule once inside the cell. In many instances, monitoring biological activity will be useful for fusion molecules having a molecular weight of less than about 45, 40, 35, 30, 25, 20 or 15 kDa.

Illustrative misfolded fusion molecules of this invention include misfolded fusion proteins. Examples of such fusion proteins include those exhibiting a specified biological activity particularly when transduced into a target cell or group of such cells. That

activity can be measured and quantified if desired to determine transduction efficiency for a particular misfolded fusion protein relative to a suitable control molecule. Examples of such assays include those which measure cell replication, cell adhesion, oncogenic transformation, cell blebbing, cell kinesis, cell division, cell death (apoptotic and necrotic), fertilization, expression of a specified cell protein such as a receptor, transcription, immunological reactivity, protein synthesis, including translation, protein modification, and secretion; protein degradation, glycosylation, and phagocytosis. Table I below provides examples of specified proteins with biological activity that have been transduced in accord with this invention.

By the term "fusion molecule" as it is used herein is meant a transduction protein covalently linked (i.e. fused) to a desired molecule by recombinant or chemical methods. If desired, the molecule can be fused to the transducing protein through a peptide linker sequence which can include one or more cleavage sites for an enzyme such as a protease. Specified transducing proteins are discussed more fully below. The molecule of interest can be any one of a protein such as an enzyme, polypeptide, peptide, amino acid, nucleic acid (RNA or DNA), lipid, glycolipid, proteolipid, carbohydrate such as a sugar, glycan, saccharide or polysaccharide, glycoprotein, proteoglycan, a drug or other synthetic or semi-synthetic small molecule, or a hybrid thereof (e.g., an RNA-DNA hybrid molecule). The molecule can be dextrorotatory (D) or levrorotatory (L) as desired. The fusion molecule can include 1, 2, 3, 5, 8, 10, 20, 50 up to about 100 or more of such molecules as desired fused to the transducing protein (or the linker if desired). As noted, that fusion molecule is preferably misfolded in accord with this invention.

The peptide linker sequence preferably may comprise up to about 20 or 30 amino acids, more preferably up to about 10 or 15 amino acids, and still more preferably from about 1 to 5 amino acids. The linker sequence is generally flexible so as not to hold the fusion molecule in a single rigid conformation. The linker sequence can be used, e.g., to space components of the fusion molecule from each other as desired. For example, the peptide linker sequence can be positioned between the protein transduction domain and

the protein sequence, e.g., to chemically cross-link same and to provide molecular flexibility.

More particularly, each component of the fusion protein can be spaced from another component by one or more suitable peptide linker sequences, e.g., to provide specified enzyme recognition sites, particularly protease cleavage sites; or tags, provided that the peptide linker sequences do not significantly impact the function for which the fusion protein is intended.

The molecular weight of the molecule will vary depending on intended use but will generally be between about 0.1 to 100 kD or greater up to about 500 to 1000 kD. Preferably, the molecular weight of the molecule will be between about 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30, or 50 kD up to about 100 kD as determined by SDS-PAGE gel electrophoresis, sedimentation centrifugation or other suitable means. The molecular weight of the fusion molecule will vary in accord with intended use but will generally be about 5, 10, 20, 30, 40, 50, 100, 200, 500, or 1000 kDa or more as measured by centrifugation sedimentation or SDS-PAGE gel electrophoresis.

Additionally preferred fusion molecules in accord with the present invention are fusion proteins comprising the transducing protein (sometimes referred to as a transduction domain) fused to a desired protein. The protein fused to the transduction domain can be, e.g., a zymogen, an enzyme, a binding partner for another amino acid or nucleic acid sequence, an immunological molecule such as an antigen binding antibody fragment, or a structural protein. Additional fusion proteins suitable for use in accord with this invention have been described in U.S. Provisional Application No. 60/069012, entitled "Anti-Pathogen System and Methods Of Use" filed on December 10, 1997, now filed as PCT/US98/26358 and U.S. application serial no. 09/208966, and also described in U.S. Provisional Application 60/056,713 entitled, "Inducible Regulatory System and use thereof," filed on August 22, 1997, now published as PCT/US98/16887 (WO 99/10376), the disclosures of all of which are incorporated herein by reference.

Still further fusion molecules including fusion proteins which can be misfolded and used in accord with this invention have been disclosed in U.S. Patent Nos. 5,652,122, 5,674,980 and 5,670,617, the disclosures of which are hereby incorporated by reference.

Preferred misfolded fusion molecules of this invention are capable of enhancing transduction of specified cells or groups of cells including tissues and organs by about 2, 5, 10, 20, 50, 100, or about 200 fold or more when compared to transduction with a suitable control molecule, preferably a native fusion molecule corresponding to the misfolded fusion molecule of interest. Preferred methods for measuring enhancement of protein transduction are described in more detail below.

As noted, the term "misfolded" is used herein to denote a misfolded fusion molecule that has been denatured and then subjected to one or more specified conditions as disclosed herein. Without wishing to be bound to any particular theory, it is believed that when the denatured fusion molecule is exposed to the conditions it becomes energetically unstable relative to its native or near-native conformation. It is believed that the energetic instability contributes significantly to the enhanced transduction capacity of the fusion molecules. In many instances, the misfolded fusion molecules will exhibit a Gibbs free energy (ΔG) that is about 2, 3, 5, 10, 20, 50, or 100 up to about 250 fold or more higher than the ΔG of the native molecule.

The Gibbs free energy of a desired molecule can be determined by a variety of conventional thermodynamic approaches. Reference herein to a misfolded fusion molecule having a "high ΔG" means that at least in theory the molecule has a large negative Gibbs free energy and that folding of the misfold molecule can proceed spontaneously under appropriate conditions. In contrast, reference to a "low ΔG" means that the fusion molecule has at least in theory a zero or positive Gibbs free energy compared to its native conformation indicative of an essentially stable fusion molecule that will not fold (or unfold) spontaneously. See generally Edsall, J.T. and Gutfreund, H., *Biothermodynamics: The Study of Biochemical Processes at Equilibrium,* (Wiley, 1983).

For example, a preferred method for determining the Gibbs free energy involves use of the following formula: $\Delta G = -2.303RT\log k_{eq}$, wherein ΔG is the standard free energy change and k_{eq} is an equilibrium constant for misfolding a desired fusion molecule. Methods for determining the keq are well-known in the art.

It will be apparent from the foregoing that native, denatured, and misfolded fusion proteins of this invention are readily distinguishable. For example, misfolded fusion proteins are capable of significantly enhancing transduction and exhibit a theoretically higher Gibbs free energy than the same proteins in a native form.

In addition, misfolded fusion molecules can be distinguished from native or denatured proteins in a variety of ways, e.g., by viscosity and/or optical rotation spectra. See e.g., Edsall and J.T and Gutfreund, H, *supra.*

Misfolded fusion proteins of the invention can be further identified by analysis of biochemical and/or biological activity of the misfolded protein relative to the same protein in native form. As discussed above, a misfolded protein of the invention will exhibit decreased biological and/or biochemical activity, e.g. about 20%, 30%, 40%, 50%, 60%, 70%, 80% or more of a decrease of biological and/or biochemical activity relative to the same protein in native form. In some instances, a misfolded protein of the invention will exhibit a complete or essentially complete loss of biological and/or biochemical activity relative to the same protein in native form. Such analysis of decreased biochemical and/or biological activity of a misfolded fusion protein of the invention relative to the same protein in native form can be readily determined by standard assays for the particular biological or biochemical property.

A denaturing agent suitable for use in accord with this invention is any agent including a chaotropic agent which is capable of substantially disrupting intramolecular bonds (e.g., hydrogen, ionic or covalent) in a protein, polypeptide or peptide. Illustrative

denaturing agents include certain organic compounds, salts, temperature (e.g., heat), and sound (e.g., sonication). In particular, the organic compounds can include urea, particularly in a concentration of between about 4 to 8M, preferably about 8M urea, guanidinium-HCl, and certain mercaptans such as β-mercaptoethanol. Although less preferred for some applications, the denaturing agent may be a detergent such as an ionic detergent, e.g., sodium dodecyl sulfate, (SDS); as well as other detergents such as Brijj, Nonidet-P40, and Triton-X-100. Further contemplated denaturing agents include high concentrations of a salt (e.g., molar amounts) such as NaCl, KCl, and the like.

A solution suitable for forming the misfolded fusion molecules of this invention is one which supports formation of a misfolded fusion molecule. Typically, the solution will be an aqueous buffer (or more than one of such a buffer) such as those generally known in the field. Particular examples of such buffers are described below and in Ausubel et al., *Current Protocols in Molecular Biology,* John Wiley & Sons, New York (1989), Sambrook et al., *Molecular Cloning: A Laboratory Manual.* (2nd ed. (1989), and *Harlow and Lane Antibodies*: A Laboratory Manual, CSH Publications, N.Y. (1988).

More specifically, suitable aqueous solutions referenced herein typically have a buffering compound to establish pH (e.g., Tris or Hepes), typically within a pH range of about 5-9, and an ionic strength range between about 2mM and 500mM. Sometimes a protease inhibitor or mild non-ionic detergent is added. Additionally, a carrier protein may be added if desired such as bovine serum albumin (BSA) to a few mg/ml. Further illustrative aqueous buffers include standard phosphate buffered saline, tris-buffered saline, Hepes-buffered or other well known buffers and cell media formulations.

As noted above, a misfolded fusion molecule of the present invention can be made by one or a combination of strategies. For example, a preferred method of misfolding a fusion molecule is by one or more of the following steps a) to c) as follows:

a) contacting a native (or near-native) fusion molecule with one or more denaturing agents under conditions which denature the fusion molecule,

b) applying the denatured fusion molecule to a first chromatographic implementation (e.g., an affinity column) sufficient to bind the denatured fusion molecule to the implementation in the presence of the denaturing agents,

c) eluting the denatured fusion molecule from the first chromatographic implementation under conditions which concentrate the denatured fusion molecule, and then carrying out at least one of the following three methods (i.e. method 1) or method 2a)-2d) or method 3))

1) dialyzing the denatured fusion molecule,

or

2a) applying the concentrated denatured fusion molecule to a second chromatographic implementation (e.g., an ion exchange column) sufficient to bind the fusion molecule to the implementation in the presence of the denaturing agents,

2b) contacting the bound fusion molecule with a first eluting solution comprising a step gradient comprising an aqueous buffer. The aqueous buffer is preferably sufficient to decrease or eliminate presence of the denaturing agents and to form the misfolded fusion molecule,

2c) eluting the misfolded fusion protein from the second column by contacting the fusion molecule with a second eluting solution comprising a salt gradient sufficient to release the fusion molecule from the second column; and optionally

2d) applying the eluted misfolded protein to a third chromatographic implementation (e.g., a desalting column) sufficient to reduce the salt contacting the misfolded fusion protein,

or

3) applying the denatured fusion molecule to a chromatographic implementation sufficient to eliminate the denaturing agent, e.g. elution through as desalting column.

The method is schematically shown in Figure 3.

Preferably, the misfolded fusion molecule is purified as described and is provided as a substantially pure preparation (preferably sterile) suitable for pharmaceutical or research use. Further preferred in the method are specified denaturing agents, eluting solutions, and gradient conditions disclosed in the examples which follow.

Particularly preferred misfolded fusion molecules made by the one or more of the above steps a) to c) are misfolded fusion proteins. The misfolded fusion proteins can be made in accord with the method by introducing a nucleic acid encoding the fusion protein into host cells in suitable media under conditions sufficient to express the fusion protein in the cells as an insoluble and/or soluble fraction. The insoluble fraction can include inclusion bodies. Generally, the nucleic acid can be introduced into the host cells by any acceptable method, e.g., transformation, biolistic transfer, or infection using a suitable recombinant vector. Examples of such vectors are described below. See Example 1. The fusion protein is isolated from the cell fraction by contact with one or more denaturing agents such as urea or guanadine sufficient to denature the fusion protein. The denatured fusion protein is then subjected to one or more of steps b) through g) above sufficient to form the misfolded fusion protein.

Preferably, the misfolded fusion protein is provided as a sterile and substantially pure preparation suitable for pharmaceutical or cell-culture use.

A variety of conventional chromatographic implementations are suitable for use in accord with this invention including but not limited to affinity, immunoaffinity, ion exchange, sizing, and desalting columns.

Another preferred method for producing a misfolded fusion protein includes one or more of the following steps:

a) introducing a nucleic acid encoding a fusion protein into host cells in media under conditions sufficient to express the fusion protein in the host cells,

b) preparing a fraction from the host cells,

c) isolating the fusion protein from the fraction in the presence of one or more denaturing agents sufficient to denature and solublize the fusion protein,

d) contacting the denatured fusion protein with a solution, preferably an aqueous solution; and

e) forming the misfolded fusion protein in the solution from the denatured fusion protein.

Formation of the misfolded fusion protein in the steps a) to e) above can be performed by any of the methods described herein. As noted above, the cell fraction can be insoluble, soluble, or it can include insoluble and soluble cell constituents.

Although not necessary to practice the present invention, misfolding of a fusion molecule can be analyzed and further confirmed if desired by a variety of conventional techniques including e.g. performing circular dichroism, kinetic folding analysis, optical spectroscopy, NMR spectroscopy (including solution NMR) and X-ray crystallography. Standard calorimetric measurements can also be performed if desired. Other known methods for making structural determination of proteins also can be employed as will be appreciated to those skilled in the art upon consideration of the present disclosure. See, for example, methods disclosed in C. Tanford, *Physical Chemistry of Macromolecules* and other references cited herein.

Additionally, preferred misfolded fusion molecules of the present invention are fully soluble in aqueous solution. By the term "fully soluble" or similar term is meant that the fusion molecule and particularly a fusion protein that is not readily sedimented under low G-force centrifugation (e.g. less than about 30,000 revolutions per minute in a standard centrifuge) from an aqueous buffer, e.g., cell media. Further, the fusion molecule is soluble if the it remains in aqueous solution at a temperature greater than about 5-37°C and at or near neutral pH in the presence of low or no concentration of an

anionic or non-ionic detergent. Under these conditions, a soluble protein will often have a low sedimentation value e.g., less than about 10 to 50 svedberg units.

A "polypeptide" refers to any polymer preferably consisting essentially of any of the 20 natural amino acids regardless of its size. Although the term "protein" is often used in reference to relatively large proteins, and "peptide" is often used in reference to small polypeptides, use of these terms in the field often overlaps. The term "polypeptide" refers generally to proteins, polypeptides, and peptides unless otherwise noted.

As used herein, the term "cell" is intended to include any primary or immortalized cell line, any group of such cells, such as a tissue or an organ that is capable of being transduced. Preferably, the cell is of mammalian and particularly of human origin although in some cases, the cell can be derived from sheep, cow or horse. Particularly preferred are primate and more particularly human cells, tissue or organs. Examples of such cells include peripheral blood lymphocytes, whole blood cells, bone marrow stem cells, diploid fibroblasts, fibrosarcoma cells, keratinocytes, leukemic T-cells, osteocarcinomas, gliomas, murine 3T3 cells, hepatocarcinomas, and macrophages.

Also as used herein "cell" is intended to mean a cell, e.g., *E.coli* used to express a desired fusion protein. Also meant are those cells which serve as "targets" for transducing molecules of this invention.

Suitable cells can be obtained from a variety of public and commercial sources such as the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852.

A "host cell" in accord with the invention can also be a cell such as a bacterial cell (e.g., E. coli) that can be used to express a desired fusion protein. Other suitable bacterial host cells are known in the field.

As noted, the present invention pertains to fusion molecules and particularly to misfolded fusion proteins. The term fusion protein as specified above is intended to describe at least two polypeptides, typically from different sources, which are operatively linked. With regard to the polypeptides, the term "operatively linked" is intended to mean that the two polypeptides are connected in manner such that each polypeptide can serve its intended function. Typically, the two polypeptides are covalently attached through peptide bonds and can be separated by a peptide linker if desired. The fusion protein is preferably produced by standard recombinant DNA techniques. For example, a DNA molecule encoding the transduction domain is ligated to another DNA molecule encoding the protein or polypeptide, and the resultant hybrid DNA molecule is expressed in a host cell to produce the fusion protein. The DNA molecules are ligated to each other in a 5' to 3' orientation such that, after ligation, the translational frame of the encoded polypeptides is not altered (i.e., the DNA molecules are ligated to each other in-frame). The resulting DNA molecules encode an in-frame fusion protein.

The order or number of components of the fusion molecule are generally not important provided each molecule component is operatively linked and can perform specified functions for which it was intended. Fusion molecules comprising multiple components, for example more than one transducing domain or more than one covalently linked molecule are within the scope of this invention.

The transduction domain of the fusion molecule can be nearly any synthetic or naturally occurring amino acid sequence that can transduce or assist in the transduction of the fusion molecule in its misfolded form. For example, transduction can be achieved in accord with the invention by use of an HIV TAT protein or fragment thereof that is covalently linked to the fusion molecule. Alternatively, the transducing protein can be the *Antennapedia* homeodomain or the HSV VP22 sequence, or suitable transducing fragments thereof known in the field. Also contemplated are transduction domains comprising or consisting of synthetic peptides including TAT or ANTP derivatives. The fusion molecule is suitably misfolded to enhance transduction into the target cells. See

e.g., Frankel, A.D. and Pabo, C.O., *supra*; Falwell, S., et al. *supra*; Chen, et al. *supra*; U.S. Pat. Nos. 5,652,122 and PCT WO/04686.

The type and size of the transducing protein will be guided by several parameters including the extent of transduction desired. A suitable transducing protein preferably will be capable of transducing at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or even up to about 100% of the target cells when misfolded in accord with this invention. The efficiency of transduction of a particular transducing domain can be measured by one or a combination of means including measuring the percentage of transduced cells versus non-transduced cells. Particular preferred transduction domains are capable of transducing up to about 100% of the total number of targeted cells.

In most instances the efficiency of transduction of a misfolded fusion molecule will be determined by specific reference to a native or near-native form of that fusion molecule to generate activity. The determinations of transduction efficiency with respect to a misfolded fusion molecule of interest can be performed on cells or groups of such cells including tissue and organs as needed. Illustrative transduction efficiency assays are described in more detail below.

Additionally preferred transduction domains will manifest cell entry and exit rates (sometimes referred to as k_1 and k_2, respectively) on misfolded fusion proteins that favor at least picomolar amounts of the fusion molecule in the cell. The entry and exit rates can be readily determined or at least approximated by standard kinetic analysis using detectably-labeled fusion molecules. Typically, the ratio of the entry rate to the exit rate will be in the range of about 5 to 100 up to about 1000.

As noted previously, the fusion molecules of this invention can be made chemically cross-linking techniques such as those disclosed in Means, G.E. and Feeney, R.E. (1974) in *Chemical Modification of Proteins,* Holden-Day. See also, S.S. Wong (1991) in *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press. Preferably,

the fusion proteins are made by standard recombinant manipulations to make a suitable genetic in-frame fusion. See generally Ausubel et al. *supra* and Sambrook et al. *supra*

As noted, it is preferred that the misfolded fusion molecules of the present invention be substantially pure. That is, the fusion molecules and particularly fusion proteins have been isolated from cell substituents that can accompany it. Typically, the fusion molecules are present in at least 90 to 95% homogeneity (w/w). Misfolded fusion molecules and particularly misfolded fusion proteins having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified the fusion molecule should be substantially free of contaminants for therapeutic applications. Once purified partially or to substantial purity, the soluble fusion molecules can be used therapeutically, or in performing *in vitro* or *in vivo* assays as disclosed herein. Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

As is described in more detail below, fusion proteins of the present invention can be separated and purified by appropriate combination of known techniques. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electrical charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatograph, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatograph and methods utilizing a difference in isoelectric point, such as isoelectric focusing electrophoresis, metal affinity columns such as Ni-NTA. See generally Sambrook et al. and Ausubel et al. *supra* for disclosure relating to these methods. The fusion proteins are typically misfolded as described herein.

The examples below demonstrate synthesis and use of a variety of misfolded fusion proteins including over 35 full length wild type and mutant TAT fusion proteins

(see Table I). The examples also show a variety of *in vivo* biochemical and biological responses that have been scored (see Table I). Thus, it is shown that use of bacterially expressed and then misfolded, in-frame full length TAT fusion proteins effects efficient transduction into both primary and transformed cells in a concentration-dependent fashion. Fusion proteins transduced by the methods are capable of binding intracellular cognate targets and eliciting biological responses.

As noted above, the present invention substantially enhances transduction efficiency of a variety of fusion molecules. As an illustrative example, about 300 μM of a 20 amino acid p16 peptidyl mimetic can be fused to the TAT transduction domain to obtain a G_1 phase cell cycle arrest. However, the present invention only requires use of 300 nM of full length TAT-p16 protein to achieve the same effect. In addition, the p16 peptidyl mimetic induces an apoptotic side-effect, whereas the full-length protein does not. Thus, transduction of full length p16 protein (156 amino acids) into cells increases the specificity of the biological response by three orders of magnitude compared to a peptidyl mimetic.

The preferred fusion protein purification protocol described below optimizes misfolding in several ways. For example, in one embodiment, a desired TAT fusion protein was isolated under denaturing conditions sufficient to produce what is believed to be a highly energetic misfolded protein. More particularly, misfolding of the TAT fusion protein was optimized by providing "shock" misfolding conditions. That is, the TAT-fusion protein was subjected to aqueous conditions following denaturation. Figure 3 provides an outline of the "shock" misfolding process specifically described below.

The "shock" misfolding purification protocol detailed below achieved at least two goals. First, the bulk of bacterially expressed recombinant proteins were found to be insoluble and present in inclusion bodies. As noted, methods of the invention advantageously provide for the utilization of this material. Second, without wishing to be bound to any particular theory, it is believed that to traverse the cell membrane, TAT

fusion proteins require unfolding into a substantially linear array. Therefore, it is believed that use of energetically unstable fusion proteins exhibiting a high ΔG compared to correctly folded proteins in their active and hence, low ΔG stable state, appears to enhance ability to transduce a variety of cells. Without wishing to be bound to any particular theory it is believed that once inside the cell, transduced proteins become refolded by proteins such as chaperones which have been shown to refold misfolded proteins into their active state. A suitable chaperone can be Hsp90.

Figures 10a and 10b are schematic representations which illustrate the enhanced transduction efficiency provided by the present invention.

The cDNA sequences referenced herein can be obtained from a variety of public and commercial sources.

For example, one source is the National Center for Biotechnology Information (NCBI)- Genetic Sequence Data Bank (Genbank). A DNA sequence listing can be obtained from Genbank at the National Library of Medicine, 38A, 8N05, Rockville Pike, Bethesda, MD 20894. Genbank is also available on the internet at http://www.ncbi.nlm.nih.gov. See generally Benson, D.A. et al. (1997) *Nucl. Acids. Res.* 25: 1 for a description of Genbank.

More particularly, Genbank provides a variety of cDNA sequences suitable for use with the present invention. Selection of a suitable cDNA from Genbank or another appropriate source of cDNAs will be guided, e.g., by the type of fusion molecule desired to be transduced (i.e. enzyme, zymogen, nucleic acid, etc.).

The following Table A illustrates preferred methods of making shock misfolded fusion molecules as described more fully in the examples which follow. The table also points out the energetics and relative transduction efficiency of each preferred method. The terms "poor", "good" and "best" will be understood to be relative terms that are

meant to compare the methods in the table to recognized prior methods of transducing fusion proteins.

Table A

	METHOD	ENERGETICS	TRANSDUCTION
1.	Soluble protein/correctly folded	Stable/Low ΔG	Poor
2.	Denatured then Dialyzed	Unstable + Stable High ΔG/Low ΔG	Good
3.	Denatured and Shock Misfolded on Ionic Exchange Column	Unstable/ High ΔG	BEST
4.	Denatured then Shock Misfolded by Massive Dilution	Unstable/ High ΔG	BEST (theoretical)
5.	Denatured then Shock Misfolded by Rapid Desalting	Unstable/ High ΔG	BEST

All documents mentioned herein are incorporated herein by reference.

The following non-limiting examples are illustrative of the invention.

Example 1 - Production of a pTAT Fusion Protein Expression Vector

A preferred plasmid for TAT fusion protein expression has been described in the U.S. Provisional Application No. 60/06912. A map of that plasmid is depicted in Figure 1a of the drawings. Figure 1b shows the pTAT vector encoded a fusion protein comprising HIS, TAT and A protein "X" which can be any of the proteins described herein. Figure 2 shows a nucleotide sequence (SEQ ID NO:11) and amino acid sequence (SEQ ID NO:12) of the pTAT linker as well as a nucleotide sequence (SEQ ID NO:6) and amino acid sequence (SEQ ID NO:13) of the pTAT-HA linker.

pTAT and pTAT-HA (tag) bacterial expression vectors were generated by inserting an oligonucleotide corresponding to the 11 amino acid TAT domain flanked by glycine residues to allow for free-bound rotation of the TAT domain (G-YGRKKRRQRRR-G) (SEQ ID NO:14) into the BamHI site of pREST-A (Invitrogen).

A polylinker was added C' terminal to the TAT domain (see Figure 1A and 1B) by inserting a second oligonucleotide into the NcoI site (5' or N') and Eco RI site that contained NcoI-KpnI-AgeI-XhoI-SphI-EcoRI cloning sites. This LCS is followed by the remaining original polylinker of the pREST-A plasmid that includes BstBI-Hind III sites.

The pTAT-HA plasmid was made by inserting an oligonucleotide encoding the HA tag (YPYDVPDYA SEQ ID NO:15; see Figure 2 where sequence is bold) flanked by glycines into the NcoI site of pTAT. The 5' or N' NcoI site was inactivated leaving only the 3' or C' to the HA tag followed by the above polylinker. The HA tag allows the detection of the fusion protein by immunoblot, immunoprecipitation or immunohistostaining by using 12CA5 anti-HA antibodies.

The nucleotide and amino acid sequences of each linker are set forth in Figure 2. The pRSET-A backbone encodes ampicillin resistance, fl, ori, ColE1 ori (plasmid replication) and the transcript is driven by a T7 RNA polymerase promoter.

The pTAT vector contains an N' terminal 6-histidine leader followed by a minimal, highly basic 11 amino acid TAT protein transduction domain, that is flanked by glycine residues for free bond rotation, followed by a polylinker (Fig. 1a).

Example 2 - Production of TAT- Fusion Proteins

To demonstrate the utility of the pTAT construct and to show efficient transduction of full-length proteins, a series of genetic in-frame pTAT fusion vectors were made with wild-type ("wt") and an inactive tumor-derived mutant ("mut") proteins, e.g., (R87P) p16 ^{INK4a} tumor suppressor and Herpes Simplex Virus thymidine kinase (HSV-TK) cDNA. See e.g., Hall, M. and Peters, G. (1996) *Advances in Cancer Res.* 68: 67.

The pTAT vector served as a parental vector for making the fusion proteins. In general, a cDNA encoding a desired amino acid sequence was ligated into the pTAT

vector polylinker sometimes referred to as a multiple cloning SITE or "MCS" by standard means to produce an in-frame fusion protein. DH5-α bacteria were used as host cells to propagate vectors encoding the in-frame fusion proteins. BL21(DE3) pLysS cells (Novagen) were used to express the fusion proteins from the vectors. The pTAT fusion proteins were subsequently analyzed by SDS-PAGE gel electrophoresis. The production of specific TAT in-frame fusion proteins are desirable as follows:

1. TAT-p16 fusion proteins:

pTAT-p16 wild type (sometimes referred to as $p16^{wt}$) and pTAT-p16 mutant vectors were generated by ligation of an appropriate cDNA into the pTAT vector polylinker. The mutant p16 protein is sometimes referred to as $p16^{mut}$ or $p16^{INK4a}$ to designate the R87P mutation in the wild-type p16 sequence. See Hall et al., *supra. Advances in Cancer Res.* 68, 67-108 (1996). The cDNAs were individually inserted into the NcoI/EcoRI site of the pTAT vector polylinker. The cDNA sequence for the p16 protein can be obtained from Genbank. The molecular weight of the fusion proteins encoded by the vectors was about 20 kDa as estimated by SDS-page gel electrophoresis.

2. TAT-HPV E7 fusion protein (Strain HPV16):

pTAT-HPV $E7^{wt}$ and pTAT-HPV E7 mutant vectors were made by individually inserting cDNAs encoding the $E7^{wt}$ or $E7^{mutant}$ proteins into the pTAT vector polylinker (NcoI/EcoRI). The cDNA sequence for $E7^{wt}$ can be obtained from Genbank. The mutant E7 protein is sometimes referred to as $E7^{mut}$ to denote the deletion in the $E7^{wt}$ sequence of the wild-type E7 sequence. The molecular weight of the fusion proteins encoded by the vectors was about 18 kD as estimated by SDS-page gel electrophoresis.

3. TAT-13S E1A fusion protein (Adenovirus strain 5):

A pTAT-13S E1A vector were made by inserting a cDNA encoding the 13S E1A protein into the pTAT vector polylinker (NcoI/EcoRI site). The cDNA sequence encoding the 13S E1A protein can be obtained from Genbank. The molecular weight of

the fusion protein encoded by the vector was about 61 kD as estimated by SDS-page gel electrophoresis.

4. TAT-$p27^{Kip1}$ fusion protein:

A pTAT- $p27^{Kip1}$ vector was made by inserting a cDNA encoding the $p27^{Kip1}$ protein into the pTAT vector polylinker (Nco I/EcoRI). The DNA sequence of the $p27^{Kip1}$ protein can be obtained from Genbank. The molecular weight of the fusion protein encoded by the vector was about 30 kD as estimated by SDS-gel electrophoresis.

5. TAT-pRB fusion protein:

A pTAT- Rb vector was made by inserting a cDNA encoding the Retinoblastoma (Rb) protein into the pTAT vector polylinker (Nco/EcoR1 site). The cDNA sequence encoding the RB protein can be obtained from Genbank. The molecular weight of the fusion protein encoded by the vector was about 115 kD as estimated by SDS-PAGE gel electrophoresis.

6. TAT-HSV-TK fusion protein:

A TAT-HSV TK vector was made by the following method described in the Provisional U.S. Application No. 60/069012. The HSV-1 TK sequence was obtained from Genbank (Accession No. J02224). PCR primers were generated that corresponded to the N' and C' end of TK. After PCR, the DNA fragment was cut with NcoI/EcoRI and inserted into the corresponding polylinker site of the pTAT vector. The following primers were used in the method:

TK forward PCR primer (34MER):
5 'CGG GCC GGC CCC ATG GCT TCG TAC CCC TGC CAT C 3'
(SEQ ID NO: 16)

TK reverse PCR primer (39MER):
5' GGC GGG CCG GGA ATT CTC AGT TAG CCT CCC CCA TCT CCC 3'

(SEQ ID NO: 17)

The molecular weight of the TAT-TK fusion protein encoded by the vector was about 40 kDa as estimated by SDS-PAGE gel electrophoresis.

7. Preparation of TAT-CDK2 fusion protein:

The TAT-CDK2 fusion protein was made along the lines of the fusion proteins described above. The CDK2 cDNA sequence can be obtained from Genbank.

8. Preparation of Additional pTAT Fusion Proteins:

In addition to the foregoing examples of specified TAT fusion proteins, preparation and use of other fusion proteins has been described in the U.S. provisional application No. 60/069012 filed on December 10, 1997. In particular, the U.S. provisional application discloses production of fusion proteins comprising TAT and a potentially toxic zymogen, proenzyme or preproenzyme domain. As noted previously, these fusion proteins can be misfolded in accord with the present invention to enhance transduction in desired cells or groups of cells.

Example 3 - Preparation of Misfolded TAT Fusion Proteins

Several TAT fusion proteins described in Example 2 were purified from host cells and purposefully misfolded by the following protocol. The protocol is sometimes referred to herein as a "misfolding" or "shock misfolding" protocol or similar phrase. A fusion protein made in accordance with the misfolding protocol is sometimes referred to herein as "shock" misfolded fusion protein or like term.

Protein misfolding protocol: TAT fusion proteins were purified by sonication (3 bursts, 15 seconds each) of transfected BL21(DE3) pLysS cells (Novagen) obtained from a 5 hr 1 L culture. That culture was inoculated with 100 ml from an overnight culture in 10 ml of buffer A (8M urea / 20 mM HEPES (pH 7.2 (100 mM NaCl)). Cell lysates were resolved by centrifugation, loaded onto an Ni-NTA column (Qiagen) in buffer A plus

20mM imidazole. The column was then washed in 10X column volume, eluted by increasing imidazole concentration in buffer A (stepwise).

Preparation of the misfolded fusion molecule can proceed by, but not limited to, 1) dialysis, 2) desalting over a Mono Q/S column, or 3) rapid desalting.

1. Dialysis Method

1-3 ml of the denatured fusion protein was placed in a dialysis cassette (Pierce). The cassette was then placed in 4 liters of PBS at 4°C and allowed to dialyze. The PBS was changed 3 times every 4 hours for a period of about 12 to 24 hours to achieve salt reduction and misfolding. The misfolded protein was stored in 20 mM HEPES (pH 7.2)/137 mM NaCl and frozen in 10% glycerol at –80°C.

2. Mono Q/S desalting method

The denatured fusion protein was applied to a Mono-Q column on an FPLC (Pharmacia) in 4 M urea / 20 mM HEPES (pH 7.2, (50 mM NaCl)). To shock misfold the protein, the ionic exchange column was stepped from 4M urea to aqueous buffer and the protein was eluted by stepping from 50 mM to 1 M NaCl. The TAT fusion protein was eluted with the 1 M NaCl step, desalted on a PD-10 desalting column (Pharmacia) into PBS or 20 mM HEPES (pH 7.2)/137 mM NaCl and frozen in 10% glycerol at –80°C.

3. Rapid Desalting method

To achieve rapid misfolding of the fusion protein, 1 ml of the denatured fusion protein in 8M urea was applied to the PD-10 desalting column at 4°C and equilibrated in PBS. The fusion protein was desalted by elution with 5 mls of PBS to the top of the column. About 0.5ml column fractions were obtained. The fusion protein was found to be only in PBS fractions 5, 6, and 7. The urea was found in column fractions 10 through 15. The misfolded protein was stored in 20 mM HEPES (pH 7.2)/137 mM NaCl and frozen in 10% glycerol at –80°C.

The methods are summarized in Figure 3 below.

TAT fusion proteins remain highly concentrated and resistant to freeze-thaw denaturation when stored in 10% glycerol at –80^{0}C (Fig. 5, lanes 1 or 2).

Various TAT fusion protein fractions from the misfolding protocol were analyzed by conventional SDS-polyacrylamide gel electrophoresis (Fig. 4, lanes 1-10). TAT fusion proteins were found to comprise approximately 1-10% of the total bacterial cellular lysate; however, >99% is insoluble and present in inclusion bodies (see Fig. 4, lane 1). To solublize and denature TAT fusion proteins, bacterial pellets were sonicated in 8 M urea and the lysate applied to the Ni-NTA column (see Fig. 3). Column fractions comprising recombinant TAT fusion proteins were eluted by increasing the imidazole concentration in 8 M urea (Fig. 4, lines 7-10). Flow through and wash fractions from the Ni-NTA column were also analyzed (Fig. 4, lines 3 and 5).

Without wishing to be bound to any particular theory, it appears that efficient protein transduction across cell membranes requires unfolding of the protein into a linear array and that once inside the cell, transduced proteins are correctly refolded by chaperones, such as Hsp90. See e.g., Lissy, N.A., et al. (1998) *Immunity* 8:57-68. Thus, the misfolding protocol purifies TAT fusion proteins by subjecting them to "shock" misfolding from a denaturing environment into an aqueous one. The results are consistent with transduction requiring unfolding of the protein into a linear array. See Figures 4, 5 and 10.

1. Preparation of FITC-labeled fusion proteins.

FITC-labeled (fluorescent) TAT fusion proteins were generated by fluorescent labeling (Pierce) of misfolded protein according to standard methods. Labeled proteins were purified by gel purification in PBS on an S-12 column attached to an FPLC (Pharmacia). The misfolded and FITC-labeled fusion proteins were typically added directly to culture media.

Example 4 - TAT-Fusion Protein Transduction into Cultured and Primary Cells

Several TAT fusion proteins produced in Example 2 above were analyzed to determine ability to efficiently transduce into cultured or primary cells. In general, it was found that all TAT fusion proteins transduced efficiently into the cells.

About 0.1 to 100 nM of FITC-conjugated TAT-p16^{wt} or TAT-HSV-TK fusion protein was added to the culture media of Jurkat T cells and/or human whole blood cells. After about 15, 30 or 45 minutes, labeled protein was followed by flow cytometry analysis (FACS) (Figs. 6A-E; 7A, 7B). The results show rapid transduction of both fusion proteins into >99% of target cells, achieving maximum intracellular concentrations in less than 40 min (Figs. 6A, B). It was noted that a narrow intracellular concentration range existed within the population as measured by the FACS peak width between control and transduced cells. Similar results were observed with TAT-p16^{mut} FITC protein.

It was also found that TAT fusion proteins were capable of transducing into primary cells. As an example, whole blood cells were treated with fluorescein conjugated TAT-HSV-TK protein and analyzed by FACS at 1 hr post-addition (Figs. 6C, D). TAT-HSV TK-FITC protein uniformly transduced into all cells present in whole human blood, including both nucleated and enucleated cells. In addition, each of TAT-p16^{wt}, TAT-p16^{mut} and TAT-HSV TK proteins were also shown to efficiently transduce into diploid fibroblasts, fibrosarcoma cells, keratinocytes, bone marrow stem cells, peripheral blood lymphocytes (PBLs), leukemic T cells, hepatocellular carcinoma cells, osteosarcoma and NIH 3T3 cells.

In Figs. 6A-F, a concentration-dependent transduction and narrow intracellular concentration was noted as measured by nearly equivocal FACS peak widths between control and transduced cells. About 10,000 cells were analyzed for each histogram.

The following TAT fusion proteins were found to transduce efficiently into all tested cells after misfolding: TAT-HPV $E7^{WT/MUT}$protein, TAT-$p27^{Kip1}$ protein, TAT-13S EIA^{WT} protein, and TAT-pRB protein. The results show that it is possible to transduce fusion proteins from about 18 to 110 kD into all cells of the population.

The following Table 1 shows biochemical and biological responses induced by transduction of misfolded TAT fusion proteins misfolded in accord with the invention.

Table 1. Biochemical and Biological Responses Induced by Transduction of TAT Fusion Proteins.

TAT Fusion Protein	*In vivo* Biochemical Effect	Biological Effect
TAT-$p16^{INK4a}$	Bind Cdk6	GI arrest and Protection from TCR Induced apoptosis
TAT-$p27^{KIP1}$	Bind Cdk2	Cell Migration and G1 arrest
TAT-HPV E7	Bind pRB	Protection from TCR induced apoptosis
TAT-13S EIA	Bind pRB	ND[a]
TAT-CDK2 Dom.-Neg.	ND	Gi arrest
TAT-HSV TK plus Acyclovir	ND	Cell Killing
TAT-HIV Protease	In-vivo substrate cleavage	ND

a. ND = not determined.

1. Cell culture and flow cytometry methods $p16^{INK4a}$(-) Jurkat T cell culture conditions and centrifugal elutriations were performed as described and fractions containing G_1 phase cells were replated at ~1 x 10^6 cells/ml. See Lissy, N.A., *TCR-antigen Induced Cell Death (AID) Occurs From a Late G_1 Phase Cell Cycle Check point.* (1998). Dowdy, S.F., (1997). *Synchronization of Cells by Elutriation: Analysis of Cell*

Cycle Specific Gene Expression. In: Human Genome Methods, K.W. Adolph (ed.), (in press) (1997). Cell cycle position was determined by staining EtOH fixed cells in propidium iodide and analyzed by flow cytometry (FACS; Becton Dickinson). Uptake of FITC-labeled TAT-p16, -HSV TK, -E1A proteins in Jurkats and/or whole blood cells, was determined by FACS analysis of 10,000 live cells. Confocal microscopy was performed on paraformaldehyde fixed Jurkats transduced with TAT-p16-FITC and TAT-HSV TK-FITC conjugated proteins.

Example 5- Concentration Dependent Transduction of TAT Fusion Proteins

To determine if protein transduction occurs in a concentration-dependent fashion, fluorescein conjugated TAT-p16 and TAT-HSV TK proteins were added to Jurkat T cells at final concentrations of 4 nM, 16 nM, 40 nM and 0.25, 1.25, 10 nM, respectively, and analyzed post-equilibrium (1 hr post-addition) by FACS (Figs. 6E and 6F). Both TAT-p16-FITC and TAT- HSV TK-FITC proteins showed a concentration dependence for transduction, demonstrating the ability to modulate the intracellular concentration of transduced proteins. In addition, washing and replating of cells pre-loaded with TAT fusion proteins results in the transduction out of the cell in a concentration-dependent manner.

Example 6 - Confocal Microscopy of Transduced TAT-p16 Fusion Protein

Figs. 7A and 7B show confocal microscopy analysis of transduced TAT-p 16-FITC fusion protein. The results demonstrate presence of the fusion protein in the nucleus and cytoplasm of the cell and not merely attached to the cellular membrane. Intracellular fluorescence of TAT-p 16^{wt}-FITC protein and the absence of pericellular membrane ring staining was observed (see Figs. 7A-B).

Example 7 - Detection of Folded TAT-Fusion Proteins In Transduced Cells

Formation of complexes between binding proteins is usually optimal when the proteins are correctly folded (i.e. in a native or nearly native conformation). This

principle was used to demonstrate that transduced TAT-p16 and TAT-HSV E7 fusion proteins were correctly re-folded following transduction into target cells.

1. Labeling and immunoprecipitations

Jurkat T cells were labeled in the presence of TAT-p16 proteins for 4 hr with 250 μCi^{35} -methionine (NEN) and immunoprecipitations were performed as described with primary anti-p16, anti-E7, anti-E1A antibodies followed by secondary immunoprecipitation with polyclonal anti-Cdk6 or anti-pRb antibodies (Santa Cruz Biotechnology).

In Figs. 8A and 8B, lysates of transduced cells were immunoprecipitated with anti-p16 antibodies, re-immunoprecipitated with anti-Cdk6 antibodies and then separated by SDS-PAGE gel electrophoresis.

1. Transduced TAT-p16 fusion protein

$p16^{INK4a}$ protein has previously been shown to bind Cdk6, while tumor derived mutants, such as $p16^{mut}$ (R87P) used here, lose this property. See e.g., Hall et al., *supra*, Koh, J. et al., *Nature* 375, 506; and Lukas, J. et al, (1995). *Nature* 375, 503 (1995), Medema, R. H., (1995) PNAS 92, 6289. 10 nM and 200 nM TAT-$p16^{WT/MUT}$ fusion proteins were transduced into p16(-) Jurkat T cells for 4 to 6 hours during concomitant S-methionine labeling of cellular proteins. TAT-p 16:Cdk6 complexes were immunoprecipitated from whole cell lysates with anti-p16 antibodies followed by re-immunoprecipitation with anti-Cdk6 antibodies and resolution on SDS-PAGE (Fig. 8A). The co-immunoprecipitation and, hence, *in vivo* association of TAT-$p16^{WT}$ with Cdk6 was readily detectable at 10 nM and increased dramatically at 200 nM, while 200 nM TAT-$p16^{MUT}$ protein failed to associate with Cdk6.

2. Transduced TAT-HPV E7 fusion protein

p16 Jurakat T cells were labeled with ^{35}S-methionine for 4 to 6 hours and transduced with TAT-13SE1A ^{mut} protein or TAT- 13S $E1A^{wt}$ protein.

Immunoprecipitation of the transduced and labeled cells showed the ability of TAT-13S E1A protein, but not TAT-E7 mutant protein, to associate with the retinoblastoma tumor suppressor protein (pRb) *in vivo* (Fig. 8B). These observations indicated that transduced (misfolded)proteins are correctly refolded by chaperones once inside the cell and then are capable of associating with cognate target proteins *in vivo*.

Example 8 - Cell Cycle Arrest By Transduced TAT-p16 Fusion Protein

Transient transfection of wild type p16 expression vectors into cells results in G_1 phase cell cycle arrest. See e.g., Koh, J. et al., *supra* and Lukas, J. et al *supra*, Medema, R.H., *supra*. Therefore, to test for a biological effect of transduced TAT fusion proteins, G_1 phase specific cellular populations of p16(-) Jurkat T cells were centrifugally elutriated, replated, transduced with 150 nM and 300 nM TAT-p16^{WT} or TAT-p16^{MUT} fusion proteins and analyzed for cell cycle position by propidium iodide DNA staining and FACS analysis 16 hr post treatment (Figs. 9A-E). See Lissy, N.A., *supra*, Dowdy, S.F., (1997), *supra*. Centrifugally elutriated G1 phase Jurkat T cells were treated with 150 nM or 300 nM TAT-p 16^{wt} or TAT-p16^{mut} proteins for 16 hr then analyzed for cell cycle position by propidium iodide DNA staining and FACS. Note the transition of control and TAT-p16^{mut} treated cells from G1 into S/G2/M phases and the concentration dependent G_1 arrest of TAT-p16^{wt} treated cells.

As shown in Figs. 9A-E, Jurkat T cells transduced with TAT-p16^{WT} protein remained arrested in the G_1 phase of the cell cycle in a concentration-dependent fashion, while cells treated with TAT-p 16^{WT} protein or untreated control cells progressed from G_1 into $S/G_2/M$ phases. In addition, it has been shown that p16(+) human HaCaT keratinocytes are also arrested by TAT-p16^{WT} protein. See Ezhevsky, S.A., (1997). *Proc. Natl. Acad. Sci.* USA 94, 10699. These observations demonstrate that a transducible TAT-p16^{WT} protein retains all or nearly all of its previously known properties.

Example 9 - Increased Transduction Efficiency of Shock Misfolded Proteins

The transduction efficiency of a desired fusion protein can be determined at various steps in the misfolding protocol. See Example 3 and Fig. 3. For example, it is possible to estimate the transduction efficiency after solubilization of the fusion protein from host bacteria, after denaturation and renaturation of the fusion protein, or after denaturation of the fusion protein followed by ion exchange chromatography. Estimation of transduction efficiency is accomplished, e.g., by measuring one or more of transduction rate, intracellular amounts of the fusion protein or amounts or activity of transduced proteins compared with a suitable control.

A related experiment was performed with the TAT-p27 fusion protein. TAT-p27 was measured by ability to induce cell cycle arrest and ability to induce cell scattering in HepG2 cells after 24 to 48 hours. The cell scattering was measured by microscopy. In this experiment it was found that the denatured and shock misfolded TAT-p27 fusion protein transduced the cells greater than 20 fold more efficiently than a denatured and dialyzed TAT-p27 fusion protein.

Briefly, BL21(DE3) pLysS cells (Novagen) were transformed with the TAT-p27 vector described above and propagated for 5 hours in a 1 L culture. That culture was inoculated with 100 ml from an overnight culture. That culture was split into two fractions one of which was denatured and dialyzed as described above and one which was shock misfolded. To shock misfold the fusion protein, an aliquot was mixed and sonicated in about 10 ml of buffer A (8M urea / 20 mM HEPES (pH 7.2 (100 mM NaCl)) to form a "denatured" fraction. That denatured fraction was clarified and then loaded onto an Ni-NTA column (Quiagen) in buffer A plus 20 mM imidazole. The column was then washed in 10X column volume, eluted by increasing imidazole concentration in buffer A (stepwise) and then applied to a Mono-Q column on an FPLC (Pharmacia) in 4M urea / 20 mM HEPES (pH 7.2, (50 mM NaCl)). The resulting "denatured and shock" misfolded TAT-p27 fusion protein was eluted, desalted and stored as described in Example 2.

It was found that the denatured and shock misfolded p27 fusion protein was greater than 20 fold more efficient in transducing cells and in inducing cell scattering then the denatured and dialyzed fusion protein.

The results show that the shock misfolding protocol can produce misfolded TAT fusion proteins that have a greater than 2, 5, 10, 20, 50, 100 to 200-fold better transduction efficiency than soluble, completely or partially correctly folded protein.

The present invention can be used to enhance expression of vectors, proteins and/or assist in transduction of pharmacologically important peptidyl mimetics to target cells. The present invention overcomes problems due to low percentage of cells targeted, overexpression, size constraints and bioavailability. In one embodiment, the invention provides an in-frame bacterial expression vector, pTAT, incorporating an N' terminal protein transduction domain from HIV TAT. Also provided is a purification protocol that yields energetically unstable, misfolded proteins capable of efficient transduction. Full length TAT fusion proteins from 15 to 115 kD were transduced into >99% of all target cells examined, including: peripheral blood lymphocytes, whole blood cells, bone marrow stem cells, diploid fibroblasts, fibrosarcoma cells, leukemic T-cells, osteocarcinoma cells, gliomas, murine 3T3 fibroblasts, hepatocarcinomas, macrophages and keratinocytes. Transduction occurs in a concentration-dependent manner, achieving maximum intracellular concentrations in less than 30 minutes. Thus, transduction of proteins directly into cells has broad potential in manipulation of experimental systems and in delivery of pharmacologically relevant proteins.

Example 10- Rapid desalting method of producing shock misfolded fusion proteins

To achieve a misfolded/denatured state, TAT fusion protein was purified on Ni-NTA in 8M urea as above in the examples above. The denatured TAT fusion protein was

injected onto a PD-10 Sephadex desalting column (Pharmacia) equilibrated in PBS. The protein was separated from the urea by size exclusion. It was found that the denatured TAT fusion protein goes very rapidly from a denaturing environment (high concentration of urea) to an aqueous environment (relatively low concentration of urea). The result is that the TAT fusion protein becomes misfolded, present in a high ΔG state, and is fully soluble in aqueous solution. It is believed that rapid transition from the denaturing environment to the aqueous environment prevents formation of the normal (ie. native) structure, including alpha helices and β–pleated sheets.

The present rapid desalting method was easy to perform and could be practiced with a minimum investment of resources. It is believed that the method is generally applicable to most if not all fusion proteins.

Example 11-Analysis of Native and Misfolded Fusion Proteins

Previous attempts to harness the potential of protein transduction have relied on methodologies that used correctly folded, soluble proteins (theoretically low ΔG) and/or in combination with drugs such as chloroquine to treat the cells. In this example, transduction efficiency between native and misfolded fusion proteins is compared. Transduction of full length, wild type $p27^{kip1}$ protein (TAT-$p27^{wt}$) induces a biological response of cell migration or cell "scattering". The ability of bacterially produced soluble, correctly folded TAT- $p27^{wt}$ protein versus 8 M urea denatured, misfolded TAT-$p27^{wt}$ protein, shock misfolded on a Mono-S ionic exchange column to induce cell migration was compared (Fig. 12). Transduction of denatured, misfolded TAT-$p27^{wt}$ protein readily induced cell migration at 50 nM, 100 nM and 150 nM concentrations; however, soluble, correctly folded TAT-$p27^{wt}$ protein failed to induce cell migration at even the highest concentration. It is thus we concluded that utilization of misfolded TAT fusion proteins enhances the ability to transduce produces into cells and to modulate biological processes.

Figures 11a-e are explained in more detail as follows:

Direct comparison of soluble (SL), correctly folded TAT- $p27^{wt}$ versus denatured (DN), misfolded TAT-$p27^{wt}$ protein to induce cell migration. Cell migration determined as morphological alterations at cell membrane (induction of "puzzle-piece" morphology) and migration of cells in a colony away from each other. Positive Hepatocyte Growth Factor (HGF) and negative PBS controls (ctrl) as indicated.

This invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

All references disclosed herein are fully incorporated by reference.

What is claimed is:

1. A method of transducing a cell with a fusion molecule, the method comprising, misfolding the fusion molecule; and contacting the misfolded fusion molecule to the cell under conditions sufficient to transduce the fusion molecule into the cell.

2. The method of claim 1, wherein the misfolding step comprises subjecting the fusion molecule to a first condition sufficient to the denature the fusion molecule.

3. The method of claim 2, wherein the misfolding step further comprises subjecting the denatured fusion molecule to a second condition sufficient to form the misfolded fusion molecule.

4. The method of claim 3, wherein the misfolded fusion molecule has a higher Gibbs free energy (ΔG) than the fusion molecule in its native or near-native conformation.

5. The method of claim 4, wherein the Gibbs free energy (ΔG) is between about 2 to 100 fold or more greater than the fusion molecule in its native or near-native conformation.

6. The method of claim 2, wherein the first condition comprises contacting the fusion molecule with one or more denaturing agents.

7. The method of claim 6, wherein the denaturing agents are selected from the group consisting of an organic compound, a salt, a detergent, temperature or sound.

8. The method of claim 7, wherein the organic compound is selected from the group consisting of urea or a guanidinium salt.

9. The method of claim 3, wherein the second condition comprises contacting the denatured fusion protein with a solution sufficient to form the misfolded fusion molecule.

10. The method of claim 3, wherein the first condition comprises contacting the fusion molecule with one or more denaturing agents sufficient to denature the fusion molecule and the second condition comprises contacting the denatured fusion molecule with one or more aqueous buffers sufficient to increase the Gibbs free energy (ΔG) of the denatured fusion molecule.

11. The method of claim 10, wherein the second condition increases the Gibbs free energy (ΔG) of the denatured fusion molecule between about 2 to 100 fold or more relative to the fusion molecule in its native or near-native conformation.

12. The method of claim 10, wherein each of the contacting steps is performed with one or more chromatographic implementations.

13. The method of claim 12, wherein the chromatographic implementations comprise at least an affinity column and an ion exchange column.

14. The method of claim 13, wherein the method further comprises a desalting column.

15. The method of claim 10, wherein the contacting step of the second condition comprises diluting the denatured fusion molecule in an aqueous buffer sufficient to increase the Gibbs free energy (ΔG) of the denatured fusion molecule relative to the fusion molecule in its native or near-native conformation.

16. The method of claim 15, wherein the denatured fusion molecule is added to the aqueous buffer in a ratio of between about 1:10 (v/v) and 1:100,000 (v/v).

17. The method of claim 15, wherein the aqueous buffer is sufficient to reduce or eliminate presence of the denaturing agents.

18. The method of claim 16, wherein the method further comprises use of an implementation sufficient to concentrate the misfolded fusion molecule from the aqueous buffer.

19. A substantially pure misfolded fusion molecule produced by any one of the methods of claims 1-18.

20. A method of producing a misfolded fusion protein, the method comprising:

a) introducing a nucleic acid encoding a fusion protein into host cells in media under conditions sufficient to express the fusion protein in the host cells,

b) preparing an insoluble fraction from the host cells,

c) isolating the fusion protein from the insoluble fraction in the presence of one or more denaturing agents sufficient to denature and solublize the fusion protein,

d) contacting the denatured fusion protein with a solution sufficient to form the misfolded fusion protein ; and

e) forming the misfolded fusion protein in an aqueous buffer from the denatured fusion protein.

21. The method of claim 20, wherein the solution selected to increase the Gibbs free energy (ΔG) of the denatured fusion protein by between about 2 to 100 fold or more relative to the fusion protein in its native or near-native conformation.

22. The method of claim 20, wherein the solution is one or more of an aqueous buffer.

23. The method of claim 20, wherein the denaturing agents are selected from the group consisting of an organic compound, a salt, a detergent, temperature or sound.

24. The method of claim 23, wherein the denaturing agents include at least about 4 to 8M urea.

25. The method of claim 20, wherein step c) in the method further comprises use of at least one chromatographic implementation sufficient to separate the denatured fusion protein from the insoluble fraction.

26. The method of claim 25, wherein the chromatographic implementation is an affinity column sufficient to bind the denatured fusion protein thereto.

27. The method of claim 26, wherein the denatured fusion protein is contacted by a first eluting solution capable of eluting the denatured fusion protein from the affinity column.

28. The method of claim 27, wherein the first eluting solution comprises an organic compound.

29. The method of claim 28, wherein the eluting agent further comprises an imidazole gradient and about 4 to 8M urea.

30. The method of claim 20, wherein step d) of the method further comprises use of at least one chromatographic implementation sufficient to form the misfolded fusion protein.

31. The method of claim 20, wherein at least one of the denaturing agents are urea or a guanidinium salt.

32. The method of claim 30, wherein the chromatographic implementation is an ion exchange column and the denatured fusion protein is stepped from less than or equal to about 8M urea to an aqueous buffer sufficient to produce the misfolded fusion protein.

33. The method of claim 32, wherein the misfolded fusion protein is eluted from the ion exchange column by contacting the misfolded fusion protein by a second eluting solution.

34. The method of claim 33, wherein the second eluting solution is a salt gradient sufficient to elute the misfolded fusion protein from the ion exchange column.

35. The method of claim 34, wherein the salt gradient comprises from about 10 mM to about 2 M NaCl.

36. The method of claim 35, wherein the method further comprises reducing presence of salt contacting the misfolded fusion protein.

37. The method of claim 36, wherein the reduction in salt is accomplished by use of a chromatographic implementation.

38. The method of any one of claim 1 to 19, wherein the misfolded fusion molecule is capable of enhancing transduction by at least about 2, 5, 10, 50, 100 to 200 fold or more relative to a control molecule.

39. The method of claim 38, wherein the control molecule is the fusion molecule present in its native conformation.

40. The method of any one of claims 20 to 37, wherein the misfolded fusion protein is capable of enhancing transduction by at least about 2, 5, 10, 50, 100 to 200 fold or more relative to a control protein.

41. The method of claim 40, wherein the control protein is the fusion protein present in its native conformation.

42. The method of claim 1, wherein the fusion molecule comprises a protein transduction domain covalently linked to a molecule, wherein the fusion molecule has a molecular weight of between about 0.1 to 500 KD or greater.

43. The method of claim 42, wherein the molecule is selected from the group consisting of a nucleic acid, protein, polypeptide, peptide, amino acid, lipid, glycolipid, proteoglycan, proteolipid, carbohydrate, or glycoprotein.

44. The method of claim 20, wherein the fusion protein comprises a protein transduction domain covalently linked to an amino acid sequence wherein the fusion protein has a molecular weight of between about 0.1 to 500 KD or greater.

45. The method of claim 44, wherein the protein transduction domain is a protein transduction domain or other protein transduction domain.

46. The method of claim 45, wherein the other transducing protein transduction domain comprises at least amino acids 49 to 56 of TAT, synthetic domains, or ANTP.

47. The method of claim 44, wherein the amino acid sequence is a protein selected from the group consisting of p16, TK, EIA, p27, E7, GFP, Rb or a fragment thereof.

48. A substantially pure preparation of the misfolded fusion molecule produced by any one of the methods of claims 1 to 19.

49. A substantially pure preparation of the misfolded fusion protein produced by any one of the methods of claims 20 to 37.

50. The substantially pure misfolded fusion protein of claim 48 or 49, wherein the misfolded fusion protein is contacted by one or more stabilizing agents.

51. The substantially pure misfolded fusion protein of claim 50, wherein the one or more stabilizing agents is a bovine serum albumin, a glycol or dimethylsulfoxide.

52. The substantially pure misfolded fusion protein of claim 51, wherein the glycol is glycerol present in an amount between about 1 to 15% (v/v).

53. The substantially pure misfolded fusion protein of claim 52, wherein the misfolded fusion protein is stored between about -10 to -100°C.

54. A method of transducing a cell or group of cells with a fusion protein, the method comprising contacting the cell or group of cells with a misfolded fusion protein produced by any one of the methods of claims 20-37 under conditions sufficient to transduce the misfolded fusion molecule into the cell or group of cells.

55. The method of claim 54, wherein the cells are mammalian cells.

56. The method of claim 55, wherein the mammalian cells are immortalized or primary cells.

57. The method of claim 56, wherein the groups are mammalian cells comprising a target tissue or organ.

58. The method of claim 57, wherein the groups of mammalian cells are *in vivo*.

59. The method of claim 45, wherein the other transducing protein transduction domain comprises at least amino acids 49 to 56 of TAT.

60. The method of claim 20, wherein the host cells are bacterial cells and the insoluble fraction comprises inclusion bodies.

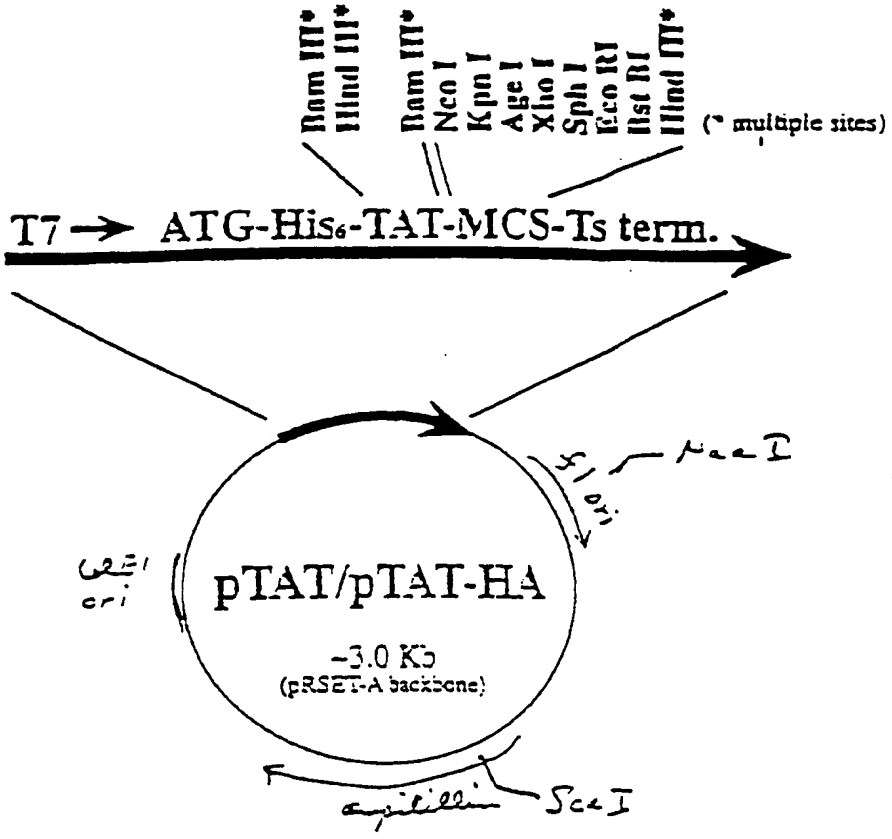

Figure 1A

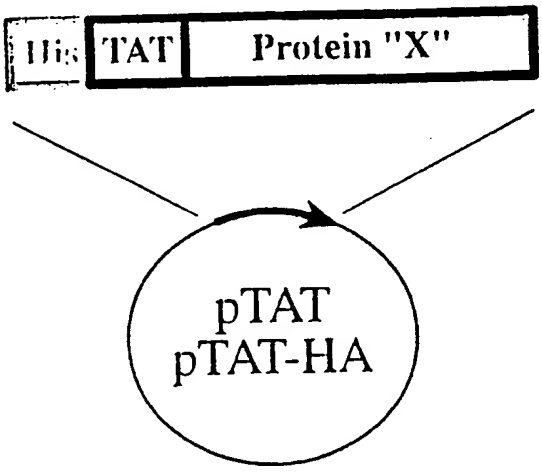

Figure 1B

pTAT LINKER:

```
 BamHI HindIII                    TAT DOMAIN
GGA TCC AAG CTT GGC TAC GGC CGC AAG AAA CGC CGC CAG CGC CGC CGC GGT
 G   S   K   L   G   Y   G   R   K   K   R   R   Q   R   R   R   G

 BamHI      NcoI      KpnI AgeI     XhoI       SphI         Ecc BstEI HindIII
GGA TCC ACC ATG GCC GGT ACC GGT CTC GAG GTG CAT GCG GTG AAT TCG AAG CTT
 G   S   T   M   A   G   T   G   L   E   V   H   A   V   N   S   K   L
```

-followed by 20 amino acids to TAA Ts termination codon.

pTAT-HA LINKER:

The HA tag, flanked by glycine residues, was inserted into the NcoI site of pTAT. The N' NcoI site has been inactivated.

```
original                                                          NEW
 NcoI-(inactive)                  AatII                           NcoI
CC ATG TCC GGC TAT CCA TAT GAC GTC CCA GAC TAT GCT GGC TCC ATG GGC ...
    M   S   G   Y   P   Y   D   V   P   D   Y   A   G   S   M   G
```

Figure 2

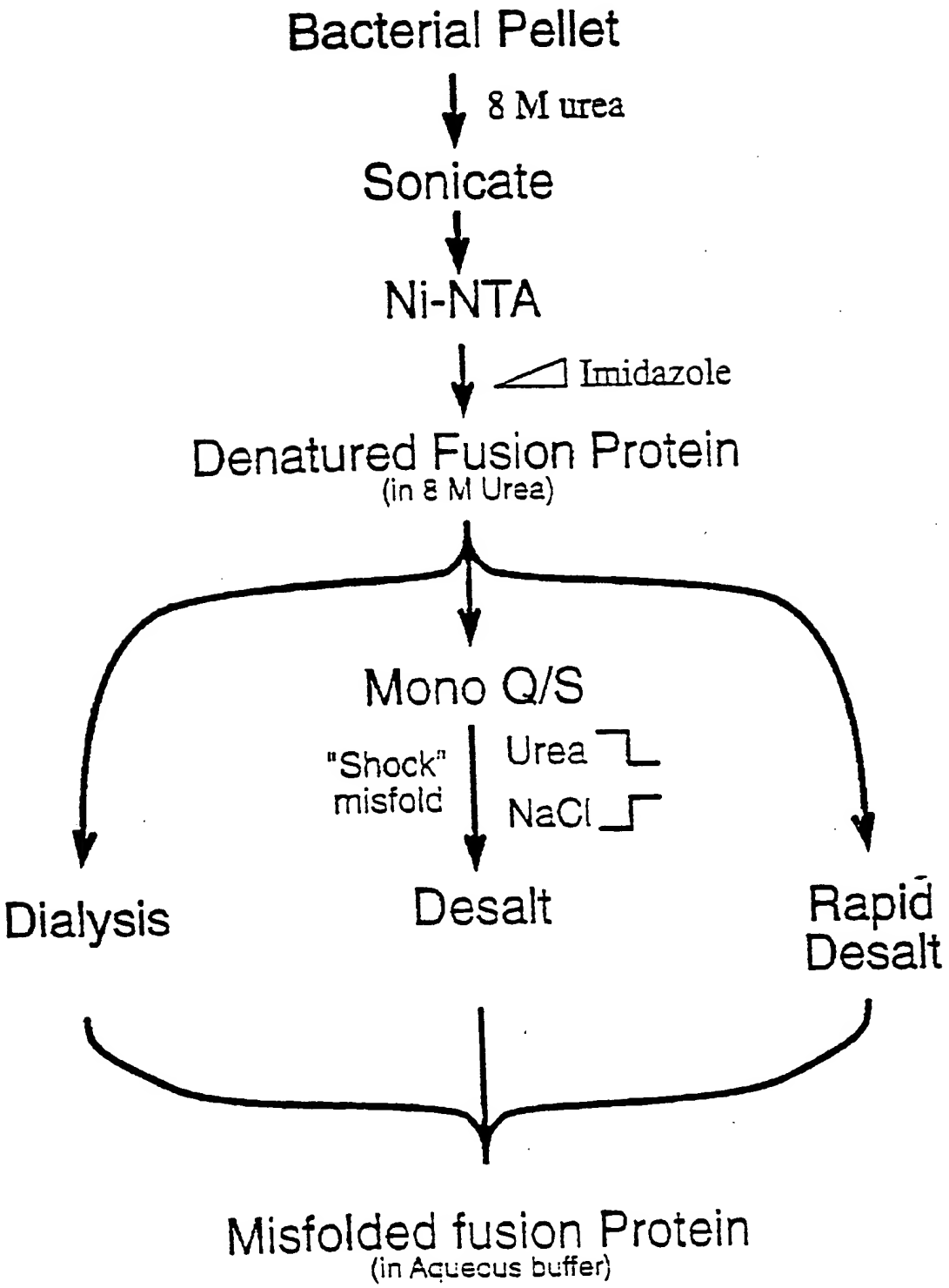
Bacterial Pellet
8 M urea
Sonicate
Ni-NTA
Imidazole
Denatured Fusion Protein
(in 8 M Urea)
Mono Q/S
"Shock" misfold
Urea
NaCl
Dialysis
Desalt
Rapid Desalt
Misfolded fusion Protein
(in Aqueous buffer)

Figure 3

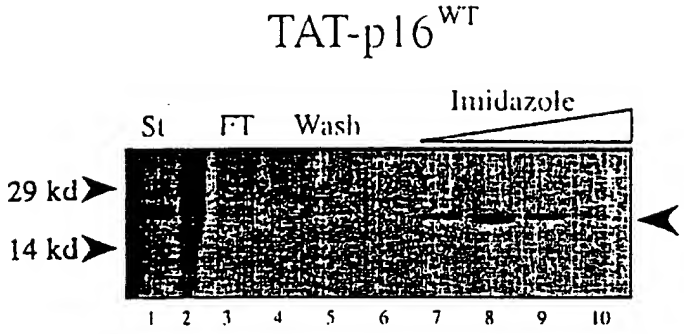

Figure 4

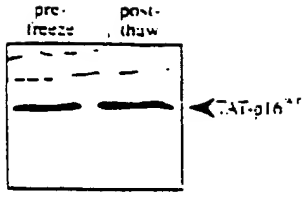

Figure 5

Figure 6A

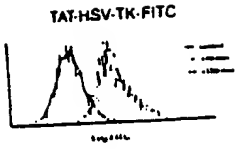

Figure 6B

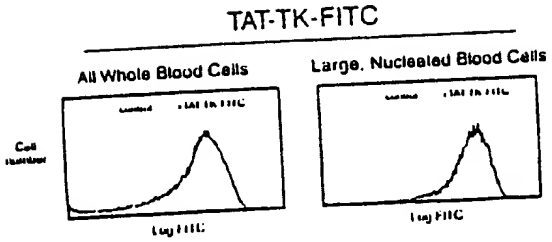

Figure 6C

Figure 6D

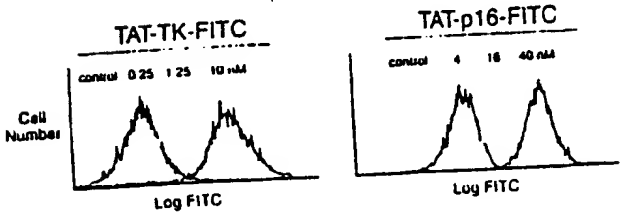

Figure 6E

Figure 6F

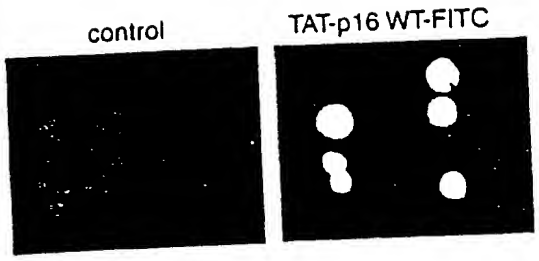

Figure 7A Figure 7B

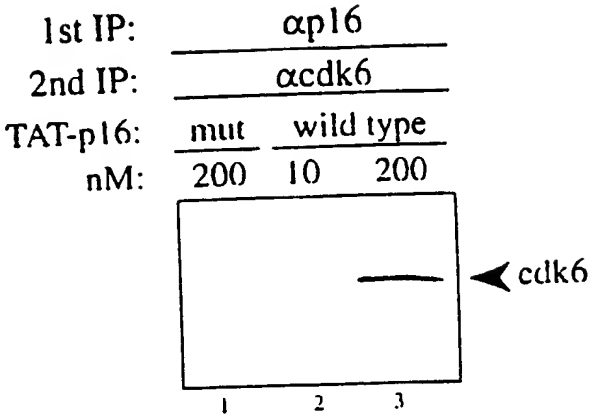

Figure 8A

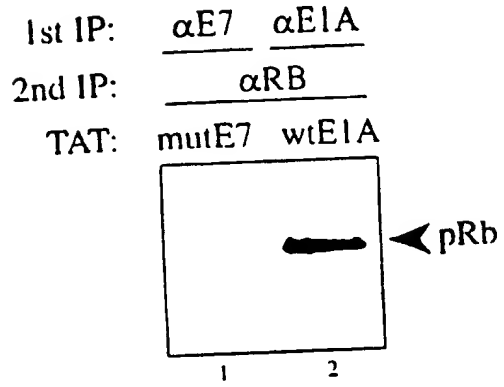

Figure 8B

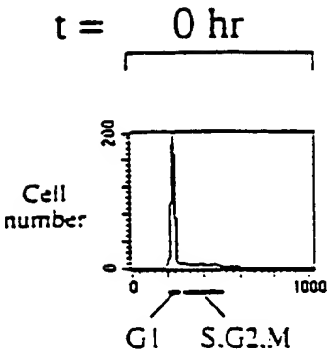

Figure 9A

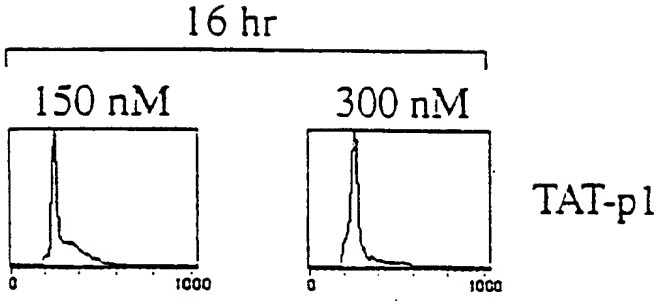

Figure 9B

Figure 9C

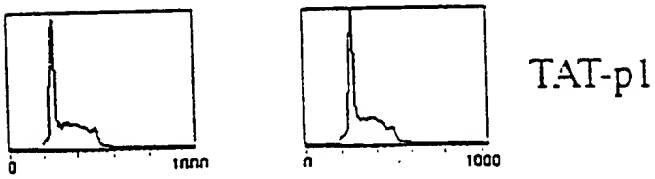

Figure 9D

Figure 9E

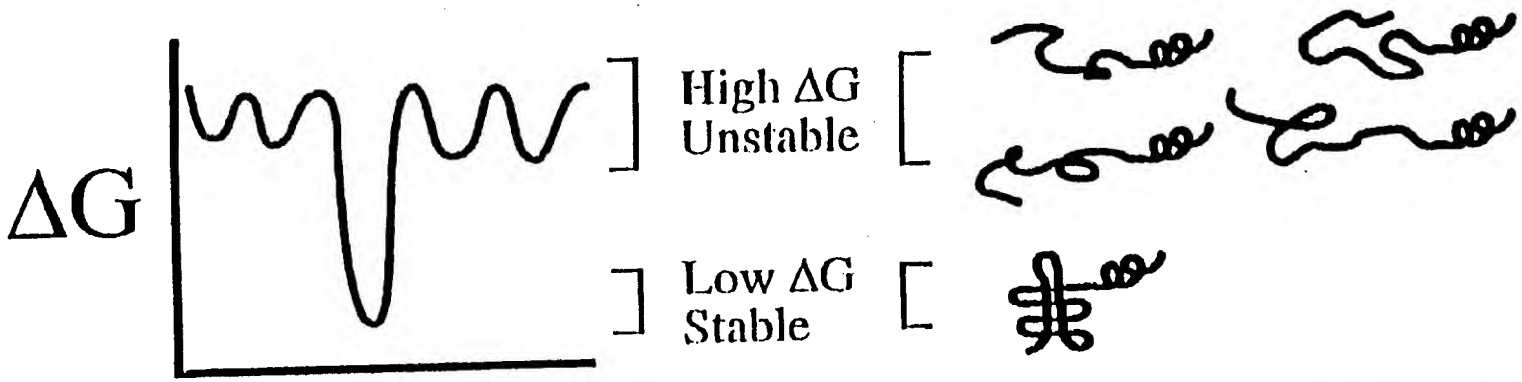

Figure 10A

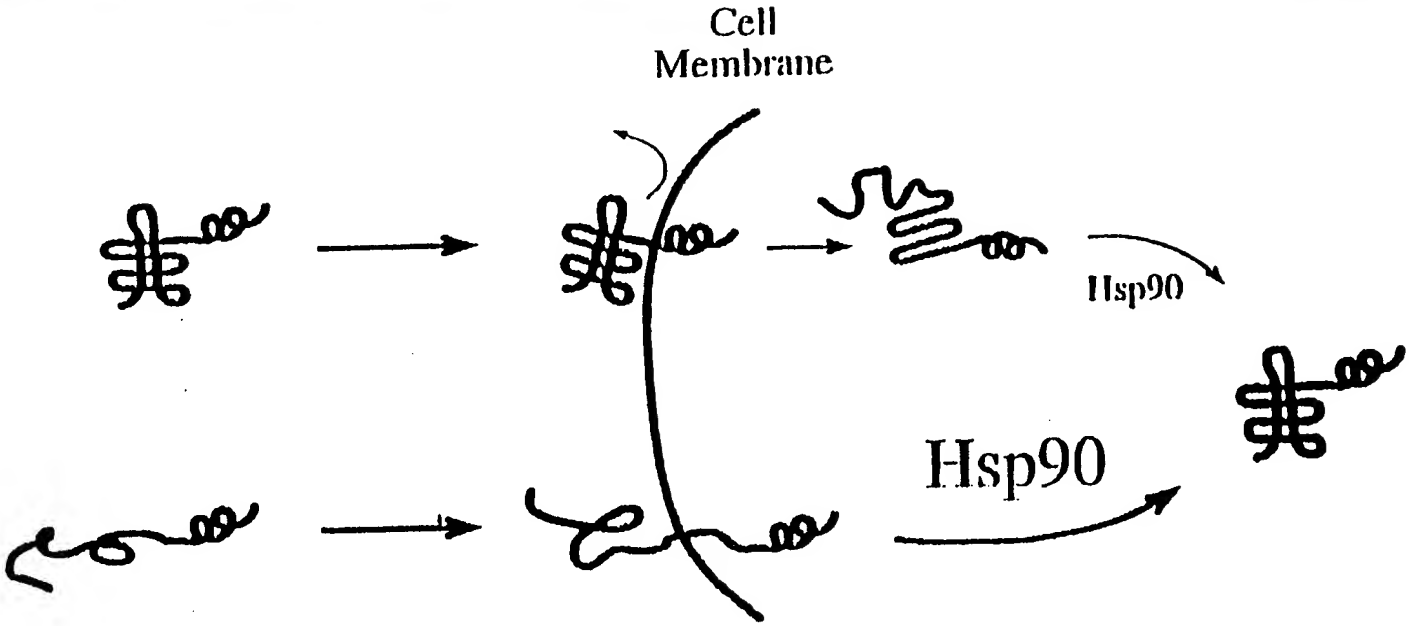

Figure 10B

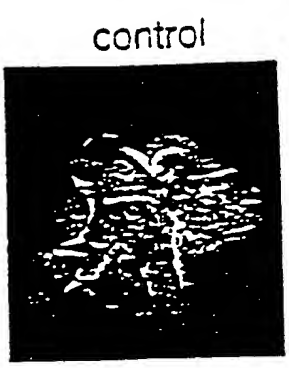

Figure 11A

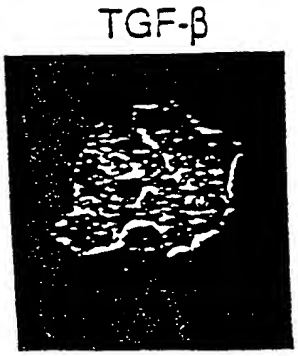

Figure 11B

Figure 11C

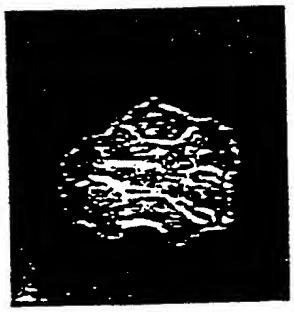

Figure 11D

Figure 11E

Figure 11F

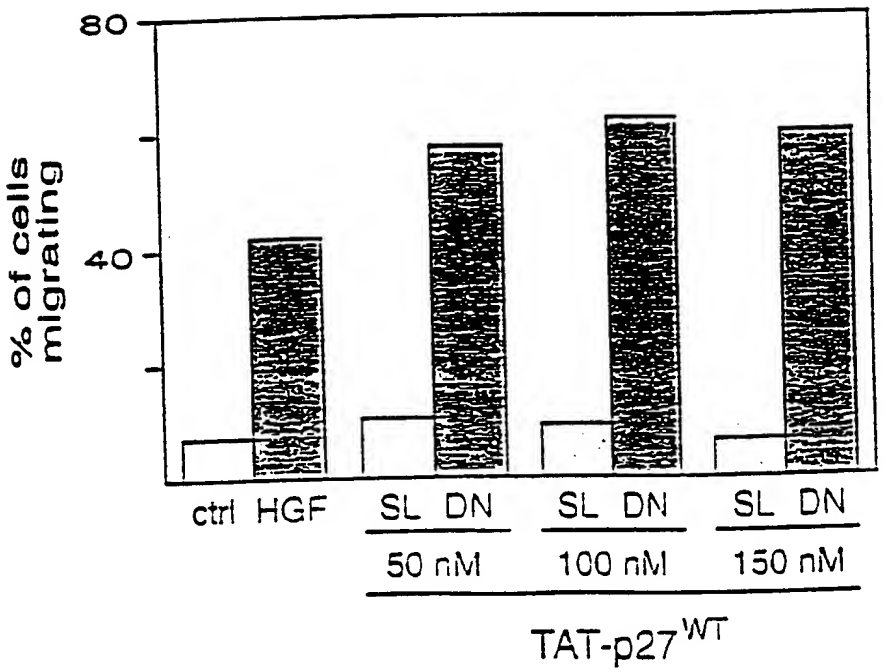
80
40
% of cells migrating
ctrl
HGF
SL DN
SL DN
SL DN
50 nM
100 nM
150 nM
TAT-p27WT

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09028

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 21/04; C12N 1/20; C07H 21/04; A61K 39/395
US CL :435/ 69.7, 252.3; 536/ 23.4; 424/134.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 69.7, 252.3; 536/ 23.4; 424/134.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS, MEDLINE, CAPLUS, EMBASE, BIOSIS, search terms: fusion, protein, transduction, unfold, misfold, transport, delivery

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STERNSON, L.A. Obstacles to polypeptide delivery. Ann. N.Y. Acad. Sci.. 1987, Vol. 507, pages 19-21.	1-18, 38-39, 42-43, 54-58,
X Y	BONIFACI, N. et al. Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding. AIDS. 1995, Vol. 9, No. 9, pages 995-1000, especially pages 995 and 999.	1, 38 2, 6-8, 40-43,48, 54-58
Y A	SAMBROOK et al. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press. 1989, pages 17.37-17.41, especially page 17.37.	20-37, 60 19, 44-53, 59

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 JULY 1999	17 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HOLLY SCHNIZER Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09028

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,652,122 A (FRANKEL et al.) 29 July 1997 (29/07/97), see entire document, especially abstract, column 18-19, and example VII.	1, 38-39, 42-46, 54-59
X – Y	US 4,999,422 A (P.M. GALLIHER) 12 March 1991 (12/03/91). see entire document, especially columns 1 and 2, and figure 1.	19-26, 31, 48-60 27-29, 32-37, 52, 53
X Y	VANDENBROECK et al. Refolding and single-step purification of porcine interferon-gamma from Escherichia coli inclusion bodies: Conditions for reconstitution of dimeric IFN-gamma. Eur. J. Biochem. 1993, Vol 215, pages 481-486, especially abstract and pages 481-482.	19, 49 20-37, 44, 45, 48, 50-60
A	VERNER et al. Protein Translocation Across Membranes. 09 September 1988, Vol. 241, pages 1307-1313, especially page 1310, column 1.	1-18, 38-39, 42-43, 54-58